Front Cover

CONTRACT NO.: DAMD 17-89-Z-9021

TITLE: Discovery and Development of Therapeutic Drugs Against Lethal Human RNA-Viruses: A Multidisciplinary Assault

PRINCIPAL INVESTIGATOR: Dr. George R. Pettit

PI ADDRESS: Cancer Research Institute
Arizona State University
Tempe, Arizona 85287-2404

REPORT DATE: August 21, 1989

TYPE OF REPORT: Midterm

PREPARED FOR: US ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK
FREDERICK, MARYLAND 21701-5014

DISTRIBUTION STATEMENT:

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official Department of the Army Position unless so designated by other authorized documents

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION / AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Arizona State University Cancer Research Institute 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) Tempe, Arizona 85287-2404	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING / SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER Contract No. DAMD17-89-Z-9021
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 62770A; PROJECT NO. 3M1-62770A871; TASK NO. AB; WORK UNIT ACCESSION NO. 374

11. TITLE (Include Security Classification)
Discovery and Development of Therapeutic Drugs Against Lethal Human RNA-Viruses: A Multidisciplinary Assault

12. PERSONAL AUTHOR(S)
Pettit, George R.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Midterm	FROM 2/6/89 TO 8/5/89	1989 August 21	65

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	,RA I; Antivirals; Natural Products; RNA Viruses; Discovery of RNA-type antiviral durgs; Naturally occurring antiviral drugs
06	03		
06	13		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The grant funds have been sharply focused on discovery of new antiviral drugs. We have been pursuing the pancratistatin family of antiviral leads as a top priority. The research results here have been very encouraging and pancratistatin, isonarciclasine, cis-dihydronarciclasine as well as trans-dihydronarciclasine have proved to be quite promising. Meanwhile over 1700 naturally occurring specimens are now undergoing preliminary antiviral evaluation at USAMRIID. The resulting leads will also be pursued as rapidly as financial resources permit. The most exciting overall result has been the discovery in USAMRIID's laboratories that pancratistatin will cure the in vivo experimental version of Japanese Encephalitis.

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION	
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL Mrs. Virginia M. Miller	22b. TELEPHONE (Include Area Code) 301/663-7325	22c. OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

X Where copyrighted material is quoted, permission has been obtained to use such material.

_____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

X Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

_____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

_____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

_____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

[signature] 8/21/89
PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

A vigorous investigation sharply focused on the isolation, characterization and structural elucidation of new and potentially useful antiviral drugs from marine animals and plants has been implemented. The program allows isolation and characterization of novel antiviral substances from confirmed active extracts of marine organisms and both higher and lower plants. Maximum effort would be devoted to marine animal and plant species yielding extracts with an outstanding confirmed level of activity in the USAMRIID antiviral screening systems. As each problem is solved (or placed in a lower priority due to decreasing or lost activity during fractionation guided by bioassay) it will be replaced by another (of high priority). Marine animals and plants represent an exceedingly productive source of new antiviral drugs and all effort will continue to be directed at making such new substances available to the U.S. Army Medical Research Institute of Infectious Diseases.

BODY

To begin with, please refer to pages 6-20 of the original proposal (see Appendix 1). That summary provides the background and direction of this research program directed very sharply at discovery of new antiviral drugs based on marine animal and plant constituents.

To summarize, a long term USAMRIID research program directed at the isolation and structural elucidation of new and potentially useful antiviral drugs from marine animals and plants is in progress. The financial support provided by the USAMRIID program will continue to be used to isolate and characterize new antiviral chemotherapeutic drugs from confirmed active extracts of marine invertebrates and vertebrates as well as marine and terrestrial plants including fungi, algae and other microorganisms. The research is sharply directed at marine animal and plant species yielding extracts with an outstanding level of antiviral activity in the USAMRIID's programs (RNA viruses).

The overall results and status to date are as follows. The grant funds are being used for discovery of new antiviral drugs. We have been pursuing the pancratistatin family of antiviral leads as a top priority. The research results here have been very encouraging and pancratistatin, isonarciclasine, cis-dihydronarciclasine as well as trans-dihydronarciclasine have proved to be quite promising. Meanwhile over 1700 naturally occurring specimens are now undergoing preliminary antiviral evaluation at USAMRIID. The most exciting overall result has been the discovery in USAMRIID's laboratories that pancratistatin will cure the in vivo experimental version of Japanese Encephalitis.

CONCLUSIONS

The discovery that pancratistatin will cure USAMRIID's in vivo Japanese Encephalitis has opened the way to a new generation of antiviral drugs. Doubtlessly, current efforts at uncovering new naturally occurring antiviral drugs will lead to analogous excellent progress.

REFERENCES

Please refer to the following manuscripts (see Appendix 2) submitted during this initial six-months of grant period, 2/6/89 - 8/5/89.

George R. Pettit, Atsushi Numata, Chika Takahashi, Tamie Miyamoto, Dennis L. Doubek, Ryoko Fujiki, and Delbert L. Herald, "Isolation and Structure of Cytostatic Steroidal Saponins from the African Medicinal Plant, Balanites aegyptiaca," Chem. Pharm. Bull. (Japan)

George R. Pettit, Atsushi Numata, Tsuruko Takemura, Richard H. Ode, A. S. Narula, Jean M. Schmidt, Gordon M. Cragg, and Charles P. Pase, "Antineoplastic Agents. 107. Isolation of Acteoside and Isoacteoside from Castilleja linariaefolia (Scrophulariaceae)," J. Nat. Prod.

George R. Pettit, Cherry L. Herald, Rajesh Gupta, John E. Leet, Daniel E. Schaufelberger, Robert B. Bates, Paul J. Clewlow, Dennis L. Doubek, Kirk P. Manfredi, Klaus Rützler, Jean M. Schmidt, Franklin B. Ward, Michael Bruck, and Fernando Camou, "Antineoplastic Agents. 168. Isolation and Structure of Axinohydantoin," Can. J. Chem.

George R. Pettit, Gordon M. Cragg, Sheo Bux Singh, James A. Duke, and Dennis L. Doubek, "Antineoplastic Agents. 162. Zephyranthes candida," J. Nat. Prod.

APPENDIX 1

3. **Body of Proposal**

a. Background

The HTLV series of RNA viruses will be used as a background illustration of the latest lethal threat to military and civilian personnel alike. Such viral diseases are now greatly intensifying the need already well established for new RNA antiviral drugs to treat the hemorrhagic fevers (e.g., Bolivian, Korean and Lassa) and encephalitises (e.g., Japanese and Venezuelan). In 1980 Gallo and colleagues at the National Cancer Institute isolated from male and female patients (originating from Georgia in the U.S. and the Caribbean) a virus designated HTLV-I for human T-lymphotropic virus or human T cell leukemia/lymphoma virus.[1] And this represented the first isolation of type-C retrovirus particles from fresh and cultured lymphocytes from a patient with T cell lymphoma.[2] Perhaps HTLV-I will also be found responsible for the elevated mortality of leukemia[3] among dairy farmers in the U.S.[4] The important discovery of HTLV-I led to the isolation of HTLV-II and HTLV-III in 1981,[5] the latter being responsible for acquired immune deficiency syndrome (AIDS) or HIV.

The leukemia viruses (retroviruses) of humans, cats and monkeys all have in common an ability to infect and transform T cells.[6,7] As just noted the general term of this family of human T lymphotropic retroviruses is HTLV or human T cell leukemia (lymphotropic) viruses.[7,8] The group includes HTLV-I, -II and -III which make up a family of exogenous retroviruses associated with adult T cell leukemia/lymphoma (ATL) and AIDS. HTLV-I has been found widely distributed on a world-wide basis and has been shown to be the direct cause of ATL. About one million people in the United States alone are infected and about one in a hundred will develop the full neoplastic disease. HTLV-II has been isolated from three patients with a T cell variant of hairy cell leukemia and also from a patient with AIDS.[9,10] The HTLV-II virus has also been found

3. Body of Proposal

a. Background

The HTLV series of RNA viruses will be used as a background illustration of the latest lethal threat to military and civilian personnel alike. Such viral diseases are now greatly intensifying the need already well established for new RNA antiviral drugs to treat the hemorrhagic fevers (e.g., Bolivian, Korean and Lassa) and encephalitises (e.g., Japanese and Venezuelan). In 1980 Gallo and colleagues at the National Cancer Institute isolated from male and female patients (originating from Georgia in the U.S. and the Caribbean) a virus designated HTLV-I for human T-lymphotropic virus or human T cell leukemia/lymphoma virus.[1] And this represented the first isolation of type-C retrovirus particles from fresh and cultured lymphocytes from a patient with T cell lymphoma.[2] Perhaps HTLV-I will also be found responsible for the elevated mortality of leukemia[3] among dairy farmers in the U.S.[4] The important discovery of HTLV-I led to the isolation of HTLV-II and HTLV-III in 1981,[5] the latter being responsible for acquired immune deficiency syndrome (AIDS) or HIV.

The leukemia viruses (retroviruses) of humans, cats and monkeys all have in common an ability to infect and transform T cells.[6,7] As just noted the general term of this family of human T lymphotropic retroviruses is HTLV or human T cell leukemia (lymphotropic) viruses.[7,8] The group includes HTLV-I, -II and -III which make up a family of exogenous retroviruses associated with adult T cell leukemia/lymphoma (ATL) and AIDS. HTLV-I has been found widely distributed on a world-wide basis and has been shown to be the direct cause of ATL. About one million people in the United States alone are infected and about one in a hundred will develop the full neoplastic disease. HTLV-II has been isolated from three patients with a T cell variant of hairy cell leukemia and also from a patient with AIDS.[9,10] The HTLV-II virus has also been found

to infect endothelial cells that line brain capillaries.[11]

The HTLV-I is now recognized as a new form of malignant T cell lymphoma characterized by hepatosplenomegaly, lymphadenopathy, skin lesions (frequently) and rapidly fatal course.[12,13] The seriousness of this first human type C-retrovirus is that HTLV-I is endemic to the southeastern United States with a rate similar to that seen in Caribbean blacks.[14,15] Of 56 Jamaicans with lymphoproliferative neoplasia 19 (34%) were found to have antibody to the HTLV virus in their sera. The majority (17) of those patients testing positive carried chronic lymphocytic leukemia or non-Hodgkin's lymphoma. When 16 consecutive patients bearing non-Hodgkin's lymphoma were examined serologically, 69% were HTLV positive.[16] In the Kagoshima, Miyazaki, Nagasaki, and Okinawa prefectures the statistically adjusted rate of death from adult T cell leukemia/lymphoma is much higher than in the rest of Japan and the sera of most patients were found HTLV-I positive.[17] A study of these patients and their migratory history suggested that the virus was endemic among Kyusu natives. Originally the HTLV-I infection was thought primarily limited to black and Japanese populations. Now the first infectious cluster has been located in a caucasian population in Italy.[18,19] Add to this the demonstration that HTLV-I is transmitted by blood transfusion and that the seroconverted recipients can then provide natural transmission (female ⇆ male, mother → child) giving rise to new HTLV-I infections leading to adult T cell leukemia/lymphoma.[20,21] The mode of action of the HTLV viruses on human T cells is really striking. The HTLV-I routinely transforms T cells while the related HTLV-III kills T cells.[22]

The HTLV-III also known as HTLV-III/LAV (for lymphodenopathy-associated virus) and HIV (for human immunodeficiency virus) has been isolated and characterized from over 95 patients (90% detection rate) with AIDS. Glimpses of related retroviruses are now beginning to appear. A retrovirus related to

HTLV-III was detected in patients with hypogammaglobulinaemia (impaired immunoglobulin production).[23] The French group of Montagnier has reported the isolation of a human retrovirus named LAV-II from two West African AIDS patients.[24] The virus was found distinct from HTLV-III/LAV. Where LAV is their equivalent of HTLV-III. At the 1986 American College of Hematology meeting Kiefer reported that a retrovirus designated B-cell leukemia-associated virus (BLAV) has been found in patients with B-cell chronic lymphocytic leukemia. Also in this area Gallo and colleagues[25,26] have now reported the isolation of a new double-stranded DNA Herpesvirus from the white blood cells of six patients (with lymphomas or other immune system disorders) designated human B-cell lymphotrophic virus (HBLV). The newly characterized virus infects and eventually kills human B cells (in vitro).

AIDS is already well established in Africa and has spread extensively in the general male and female population.[27] As of June 1986 the World Health Organization[28] estimated that some 50,000 Africans may already be ill with AIDS and that more than 6% of the population of Africa is infected with the AIDS virus.[29,30] So far the reported cases of AIDS in Africa correspond to 80% from Central and East Africa, 6% from Southern Africa and 14% from other areas. Although somewhat uneven in distribution, AIDS seems to have spread almost everywhere on the African continent.[29,30] Some conception of the extent can be ascertained from prostitutes who present at clinics for treatment. Of these 88% in Rwanda, 59% in Kenya[31] and 27% in Zaire carry the AIDS virus antibodies. In the capital of Rwanda 18% of the city population tested positive for the AIDS virus but only 3% in rural areas. The epidemiology of AIDS in Africa has been studied by Biggar of the U.S. NCI who has concluded that AIDS is presently an epidemic spreading in tropical Africa.[32] Several hundred thousand Africans are already believed to have died from AIDS and some five million may now carry the virus.

In general AIDS seems to have emerged as a problem in Africa over the past 10 years. The actual origin in Africa was probably a great deal earlier (to 1959).[33] Serological examination of specimens from African green monkeys in the United States where the samples were taken more than two decades ago proved positive for antibodies against HTLV-III.[32] The same source has been found for HTLV-I.[34] Other evidence pointing to this endemic source has come from the capture of green monkeys recently in several parts of Africa that also proved sero-positive.[35]

Heterosexual contact possibly enhanced by co-factors from venereal diseases appears to be the prime mode of HTLV-III transmission in Africa.[32] Indeed, HTLV-III was recently isolated in the U.S. from the cervical secretions of 14 women found seropositive for this virus.[36] And AIDS-associated retrovirus (ARV) was isolated in 1986 from vaginal and/or cervical secretions from four of eight women seropositive for this virus.[37] Furthermore HTLV-III has been isolated from the saliva of four ARC patients and observed by electron microscopy in a saliva preparation from one AIDS patient.[38] And the virus has also been recovered from seminal fluid[22] and tears.[39]

In May 1986 the U.S. Center for Disease Control estimated that some 100,000 (clinical estimates were 200,000) patients were suffering from the ARV retroviruses.[40] In addition to the some 25% (to 75%)[41] of the approximate 1.5 to 2 million people in the U.S. now infected with fatal AIDS another some 25% will progress to one of the ARC diseases. In the United States AIDS is about doubling each year with as just noted 1.5-2.0 million people now believed to be infected and infectious.[42]. Presently, it is generally agreed that a person producing antibodies to the AIDS virus has been infected and is probably infectious and 20-30% (to 75%)[41] will develop the full (and lethal) disease. Indeed most people may be more infectious during

early stages. Apparently when the viral genes are integrated into the cells own DNA they can remain dormant for indefinite periods. But the disease progresses when the viral genes are activated and new virus particles are formed which then infect fresh T-4 cells.[43-45] When the body's T-4 cells are seriously depleted thereby the immune system collapses and the victim appears with AIDS.

The transmission of AIDS from Central Africa[46] to Haiti and now on essentially a world-wide basis suggests that a staggering world-wide death rate from this disease will ensue unless we find drugs to control and/or cure this viral disease. Indeed antibodies to the human T cell leukemia HTLV-III virus have been found among aboriginal Amazonian Indians in Venezuela[47] and even the new "slim disease" detected in Uganda in 1986 seems associated with HTLV-III infections.[48] Very importantly a recent study at the Walter Reed Army Medical Center has provided epidemicologic evidence that AIDS occurs by both male to female and female to male transmission (bidirectional heterosexual).[49] The risk of casual contact is also real. In a recent study of household contacts with AIDS patients and controls in Kinshasa, Zaire 9.8%(20) of 204 case-household members compared to 1.9%(3) of 155 control-household members were HTLV-III seropositive. Of 18 spouses of patients with AIDS 61.1%(11) compared to 3.7%(1) of 27 control spouses were HTLV-III seropositive![50] Even more ominous was a report by Chermann[51] of the Pasteur Institute at the 14th International Cancer Congress (1986) that Central African insects such as mosquitoes, cockroaches, bed bugs, ticks and tse-tse flies are contaminated with HTLV-III! In short, the stage is now set for rapid transmission of AIDS in the United States and world-wide. Unless resources are quickly provided for the discovery of drugs that will cure and/or control AIDS phenomenal death rates and economic losses will rapidly ensue.

Since AIDS is a pandemic immunosuppressive disease, infections with opportunistic organisms and certain neoplastic diseases, particularly Kaposi's sarcoma and lymphomas lead to death generally accompanied by dementia, diarrhea, fever, generalized lymphadenopathy, neuropathy, oral candidiasis and weight loss.[52] Prospects for a vaccine[53,54] are not especially promising as there is extensive heterogeneity among various HTLV-III isolates, particularly in the env gene.[55] One hope in this direction is the recent news announcement that a major protein code of the HTLV-III virus, namely, gP120 has been synthesized by splicing its viral gene into hampster cells for production of the protein. A gene-spliced protein product might provide a useful vaccine and eliminate the need to use potentially dangerous natural virus. But if the HTLV retroviruses rapidly change their coding such vaccines would be of little long term use.[3] Furthermore, very serious questions ranging from possible future (10-15 years later) cancer onset and/or revitalization of the virus from HTLV-III and -IV based vaccines to ethical and moral considerations have been raised.

All of the HTLV viruses so far isolated and in sub-groups from HTLV-I, -II, -III have a magnesium II dependent reverse transcriptase and are T cell tropic.[9] The I-III series and bovine leukemia virus (BLV)[56] all differ from other retroviruses by a marked increase in the rate of transcription in infected as compared to uninfected cells. A complete nucleotide sequence has recently been proposed for the HTLV-III virus.[57] Almost simultaneously the French version of HTLV-III designated the LAV causative agent of AIDS was shown to have a 9193-nucleotide sequence representing the complete viral nucleotide unit.[58] Indeed, the HTLV-III and LAV nucleotide sequences differ overall by only 1%.[59] One group believes the HTLV-III, AIDS-associated retrovirus (ARV) and LAV represent different isolets of the same virus and there is only one AIDS retrovirus.[59] Thus, our group and others[60] believe

that a vigorous and well organized approach to the discovery and development of AIDS antiviral drugs will be quite successful.

The need for very effective new antiviral drugs becomes even clearer when considering that studies at the National Cancer Institute have led to isolation of HTLV-III from the cerebrospinal fluid of some AIDS patients. Gallo and Popovic have announced that the AIDS virus is also believed to infect the monocyte/macrophage cells that reside in the brain and lung. These cells appear to form the reservoir for the HTLV-III virus in the brain that enables spread throughout the body. Unlike the T cells that are eventually destroyed by the virus the monocyte/macrophage cells just keep on producing the virus and give rise to the psychoses and other AIDS-linked neurological problems. The HTLV-III virus has also been found by Wiley to infect endothelial cells that line brain capillaries.[11] Preliminary medical evidence does indicate infection of the central nervous system and this is illustrated by some patients presenting with meningitis, cortical atrophy, Guillain-Barre syndrome and motor problems in some patients not having the full blown AIDS symptoms. Other manifestations of the AIDS problem include recent evidence of patients with the AIDS virus carrying a bizarre form of Burkitt's lymphoma, Hodgkin's disease and some with squamous cell carcinoma of the head and neck.[61,62] The virtuosity of these retroviruses in destroying people is further evidenced by the recent indication of an HTLV-I relative being implicated in multiple sclerosis.[63]

As of May 4, 1987 AIDS had affected 35,219 Americans of whom 20,352 are already dead. By November 24, 1986 there were 28,169 diagnosed cases of AIDS with 15,818 already fatal.[41] The U.S. Public Health Service has now projected that in 5 years (1991) the number of Americans afflicted with AIDS would rise to 270,000 with 179,000 comprising the death toll to that period. Another 74,000 AIDS cases would be added to the total of 1991 itself and correspond to

about three times the incidence of new cases of polio in the peak year (22,000 cases) of 1952. If current predictions by a German group prove correct that 75% of people infected with HTLV-III will progress to the fatal disease in seven years instead of the presently believed 20-30% the mortality rate will increase dramatically.[41] Currently some 73% of the first 10,000 cases of AIDS in the U.S. has resulted in a 1.4 billion expenditure for medical care and an economic loss of over 4.8 billion.[64]

Presently, only a very small number of clinically active antiviral drugs are available and they include 5-iodo-2'-deoxyuridine (Idoxuridine, IDU)[65] synthesized in 1959 and now being replaced by 9-β-D-arabinofuranosyladenine (Vidarabine, ara A),[66] 5-trifluoromethyl-2'-deoxyuridine (F3TdR), 9-(2-hydroxyethoxymethyl)guanine (Acyclovir, ACV), (E)-5-(2-bromovinyl(-2'-deoxyuridine (BVDU) being discontinued due to carcinogenicity, 2'-fluoro-5-iodo-arabinofuranosylcytosine (FIAC),[67] 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Ribavirin or Virazole), 1-adamantanamine hydrochloride (Amantadine) and trisodium phosphonoformate (Foscarnet or PFA).[68,69] Except for the latter two substances these leads arose from the original marine sponge constituents β-D-arabinosyl nucleosides, spongouridine and spongothymidine.[70] A majority of present efforts to discover new antiviral drugs is being focused on further synthetic modifications of the pyrimidine and purine heterocyclic systems.[68,71-73] Presently, two of the more promising antiviral drug possibilities are Ribovirin discovered by Robins at ICN and 3'-azidothymidine (AZT)[74,75] discovered by Prusoff and Lin at Yale Univ.[68]

The antitryponosomal naphthalene sulfonate Suramin,[76] D-penicillamine,[77] Evans Blue[78], ATA[78] and phosphonoformate[68,69] (has potent side effects including kidney failure) have been found to inhibit HTLV-III replication in vitro. A clinical trial of Suramin produced serious side effects (and did not penetrate the CNS), no evidence of clinical improvement, and has been dis-

continued. A clinical trial of D-penicillamine (used to treat rheumatoid arthritis and Wilsons disease) showed suppression of HTLV-III but it also depressed the T cell levels. So far AZT (first drug to prolong the life of AIDS patients),[79,80] 2',3'-dideoxycytidine,[78] and 2',3'dideoxy-adenosine[81] appear to be the most potent and selective inhibitors of HTLV-III.[78]

Other substances or mixtures with reverse transcriptase (in vitro) or other types of activity include: AL 721, a lipid mixture (a 7:2:1 ratio of neutral glycerides), phosphatidylcholine and phosphatidylethanolamine, ampligen (a mismatched double-stranded RNA polynucleotide-induces release of interferon without side effects of injected interferons),[81] ansamycin, rifabutin (a rifamycin type antibiotic) useful against AIDS patients infected with Mycobacterium avium-intracellulare, (Mycobacterium tuberculosis also occurs at about the same rate in AIDS patients),[82] azimexon (an aziridinyl immune modulator, some improvement in ARC patients but not with AIDS), cyclosporin A (inhibits T4 lymphocyte-dependent immune responses-controversial AIDS therapy in France), HPA-23 [ammonium 21-tungsto-9-antimoniate, $(NH_4)_{18}(NaW_{21}Sb_9O_{86})_{17}$, controversial treatment used in Paris on actor Rock Hudson], imreg-1 (a proprietory immune modulator derived from white blood cells that is claimed to stimulate production of γ-interferon and interleukin-2), inosine pranobex (3:1 dimethylaminoisopropanol:inosinate p-acetamidobenzoic acid salt an antiviral and immune modulator of some use for ARC patients), and interferon alpha-2a (found to induce tumor regressions in 38% of patients with Kaposi's sarcoma).[83]

b. Hypothesis

In general the need for antiviral drugs is going to rapidly increase. Due to quick air and sea travel and transport lethal viral diseases are no longer confined to local populations in remote areas of the world. At the same time human populations are growing explosively in the more primative

countries with attendant overcrowding, malnutrition, and essentially non-existent sanitation facilities, encouraging the spread of viral diseases. And these problems are not confined to the Third-World countries. Just consider, the coastal waters of the United States are contaminated each day with five billion gallons of municipal sewage. A major portion of pathogenic viruses are associated with sewage solids and therefore not destroyed by chlorine treatment methods. At least 120 human enteric virus pathogens (e.g., hepatitis A, polioviruses, parvoviruses, adenoviruses, reoviruses) have been identified in such sewage disposed of on land or at sea. The latter viruses accumulate in bottom sediments along with the overlying sea water and enter the food chain or if on land the water tables.[84]

Unfortunately, past development and discovery of new antiviral drugs was plagued with the same general pessimism that originally surrounded the prospects for uncovering curative anticancer drugs. In the case of viruses it was felt that an effective antiviral drug would have to penetrate the cell and disrupt viral replication without affecting the cell's normal functions. That was felt to be quite unlikely and that such agents would be toxic.[85] On the other hand the extracellular replication of bacteria in body fluids was considered readily accessible to drug treatment. Fortunately present use of ARA-A for the systemic therapy of disseminated herpes simplex and zoster and for herpes simplex encephalitis[86] has dispelled such pessimistic notions and the race for improved and broad spectrum antiviral drugs is underway.[87,88]

Already, we have very strong evidence that certain marine animals and plants contain a great variety of new types of antiviral substances that should provide very useful drugs against the RNA viruses. Illustrations of this point appear in recent reference books[89,90] prepared by this writer which contain a synopsis of new antiviral, antineoplastic and cell growth inhibitory substances isolated from various plants and marine animals. Terrestrial

plants are already a well established source of new antiviral substances[91-94] and marine animals are even more promising.[10,95-97] There is great assurance that a variety of structurally diverse and useful antiviral drugs will be discovered and that such new substances will prove useful in concert against the HTLV viruses and other human pathogenic viruses. Such combination drug therapy should eliminate patterns of drug resistance that are already being observed with herpes simplex[98] patients treated with acyclovir.[99] By this means heretofore lethal viral diseases can be brought under control.

From the broader perspective one can only guess about the implication of as yet unknown viruses in the etiology of human diseases of unknown origin. For example, parvovirus infection has been found in a series of patients presenting with the clinical features of arthritis and the possibility exists that a human parvovirus could be the etiological source in some cases of rheumatoid arthritis.[100] Doubtlessly the vast reservoir of potentially useful anticancer-antiviral drugs waiting to be discovered in animal[101] and plant species[101,102-106] will eventually lead to the control and cure of human viral diseases. At the same time discovery of such new drugs[107] will allow an increase in knowledge of how to attack[108,109] human viruses and allow where needed synthetic modifications to improve efficacy.

c. Objectives

Since 1957 our research group has been completely dedicated to discovery and development of potentially useful anticancer drugs and in recent years the program has been expanded to include discovery of drugs effective against human cancer causing viruses. For example, recently we isolated a series of potentially useful antineoplastic and antiviral (unpublished) substances from plants indigenous to India, Africa, Brazil and the Caribbean. Structures have now been assigned to some of these active compounds. And a new series of structure problems will be undertaken as a very important part of the U.S.

Army Medical Research Institute of Infectious Diseases (USAMRIID) program. The very promising antineoplastic and antiviral effects exhibited by some of these new compounds and their presumably novel structures (unknown) gives every indication of additional candidates for eventual clinical trial. The recent isolation and structural elucidation of phyllanthostatins 1-3 and phyllanthoside (herpes 1 and 2 antiviral activity comparable to Acyclovir--unpublished and confidential results) provides a useful illustration of our ability to successfully complete such very important and challenging research problems.[110-112] Solution of the problem included the scale-up processing of over one quarter ton of root wood, and expertise ranging from scale-up development, bioassay (P-388 in vitro and in vivo), bioactive isolation (bioorganic chemistry), organic chemical degradation, biochemical transformation (enzymatic), carbohydrate chemistry, to structural elucidation involving techniques from high resolution mass spectrometry to 400 MHz ^{1}H-NMR, ^{13}C-NMR and x-ray crystal structure determination.

Another such recent example in the higher plant area involves our discovery[113] of pancratistatin which has exhibited a curative level of activity against the NCI murine M5078 ovary sarcoma, passed the NCI Decision Network DN2 and has shown strong preliminary activity against the bunyaviruses. Presently pancratistatin is being developed for eventual clinical trial. Meanwhile the new substance combretastatin[114] that we isolated from an African Combretum caffrum tree using the National Cancer Institute's astrocytoma brain tumor model to guide the direction of separation has subsequently been found in the USAMRIID program to have potent in vitro activity against certain RNA viruses (unpublished results). As part of pursuing the combretastatin lead to new antiviral and antineoplastic drug possibilities we have further explored Combretum caffrum for such substances

and this has just resulted in the discovery of a constituent we've designated combretastatin A-4 that displays remarkable antitubulin and cell growth inhibitory properties. A summary of the as yet unpublished combretastatin A-4 discovery and structural determination follows in 3e (Methodology Section) as an illustration of our ability to discover completely new types of antiviral drugs. Particularly significant here is the fact that tubulin has been found to be a co-factor necessary for RNA synthesis by two different classes of negative strand viruses, namely, the Sendai virus and vesicular stomatitus virus. Apparently tubulin plays a primary role in the initiation of transcription at the 3'-end of the virus genome.[115-116]

In addition to assisting the (USAMRIID) in further development of these leads support will be used specifically for the isolation, characterization, structural determination and where appropriate total synthesis of potentially useful antiviral chemotherapeutic substances from new confirmed active extracts of marine animals (invertebrates and vertebrates) and plants. Major emphasis and financial resources would be placed upon those marine animal and plant species yielding extracts with very high and reproducible antiviral activity. By way of illustration marine animal species that would first receive top priority study are those we have recently uncovered in the antiviral (RNA-types) screening laboratories of the USAMRIID. Each is now listed along with the National Cancer Institute's assignment number with antiviral <u>in vitro</u> test system and % inhibition: B 721158, 99%, YFV; B 721160, 94%, VEE; B 721166, 99%, YFV; B 721554, 99%, YFV; B 721557, 97%, VEE; B 721559, 98%, YFV; B 721562, 97%, YFV; and B 721568, 97%, YFV. As each one of these challenging research problems is solved it will be immediately replaced by another of comparable importance from our current pool of nearly 20,000 marine animal and plant species extracts that we are now beginning to evaluate for antiviral activity.

Each of the marine animal species giving confirmed active antiviral extracts was uncovered in these laboratories. Discovery of new marine organism lower plant and higher plant species bearing potentially important antiviral constituents will also be continued during the contract period. The marine animal species listed above have received no prior chemical study except in our laboratory. Also all taxonomic studies are completed in collaboration with marine zoologists of the Smithsonian Oceanographic Sorting Center, University of Cape Town, the French museum (Paris), and various university specialists in our country and abroad.

To summarize a long term USAMRIID research program directed at the isolation and structural elucidation of new and potentially useful antiviral drugs from marine animals and plants will be undertaken. The financial support provided by the USAMRIID program would be used to isolate and characterize such new viral chemotherapeutic drugs from confirmed active extracts of marine invertebrates and vertebrates as well as marine and terrestrial plants including fungi, algae and other microorganisms. The research would be sharply focused upon marine animal and plant species yielding extracts with an outstanding level of antiviral activity in the USAMRIID's programs (RNA viruses). In addition, antiparasite evaluations will be conducted by the Walter Reed Medical Research Institute. Only those species that give maximum promise of yielding new drugs with real clinical potential will be pursued as part of this USAMRIID research.

Because of 30 years spent providing the foundation for this antiviral drug discovery research a good number of exceedingly promising animal and plant species have already been uncovered as noted above and will be used along with newly discovered leads to maintain a very productive output of potentially useful antiviral substances of unique structure. Needless to say, the research proposed here would be of great assistance to the USAMRIID, the

National Institutes of Allergy and Infectious Diseases and the National Cancer Institute in selecting new antiviral and/or anticancer drug candidates and rapidly pushing their development to clinical trial.

d. Military Significance

As noted in the Background Section (a.) lethal human RNA viruses such as HIV(AIDS) pose a great and rapidly expanding threat to our military personnel. That threat is now added to the well-known lethal encephalitises, hemorrhagic fevers and a host of viral diseases of unknown etiology.

e. Methodology

Over the past thirty years the principle investigator's research program has been focused on discovery of anticancer (and in the past decade antiviral) drugs. These investigations have centered around total synthetic approaches to cytotoxic and/or antineoplastic natural products such as the amphibian venom bufadienolide constituents, the plant and animal cardenolides, and, for example, lapachol. Related efforts have been concerned with modification of natural products, such as antineoplastic antibiotics, podophyllotoxin, emetine-type alkaloids, quinones, peptides, nucleotides, and tetracyclic triterpenes, and with developing new synthetic approaches to substances generally known to exhibit anticancer activity (for example, various quinone systems). Simultaneously we have been investigating (by isolation/structural studies) natural products from plants, marine organisms, and arthropods of potential use in cancer chemotherapy. These studies, combined with related investigations in the fields of general organic synthesis, bio-organic chemistry, biochemistry and structural studies encompassing a broad area, has led this investigator to prepare over 330 original manuscripts for publication. The principal investigator has also prepared five books concerned with plant and animal products for cancer chemotherapy and seven

APPENDIX 2

Chem. Pharm. Bull (Japan)
5/7/89

ISOLATION AND STRUCTURE OF CYTOSTATIC STEROIDAL SAPONINS FROM THE AFRICAN MEDICINAL PLANT, BALANITES AEGYPTIACA[1]

George R. PETTIT,[1a] Atsushi NUMATA,[1b] Chika TAKAHASHI,[1b] Tamie MIYAMOTO,[1b]
Dennis L. DOUBEK,[1a] Ryoko FUJIKI[1b] and Delbert L. HERALD,[1a]
Cancer Research Institute and Department of Chemistry[1a]
Arizona State University, Tempe, AZ 85287-1604
U. S. A., and Osaka University of Pharmaceutical Sciences[1b]
2-10-65 Kawai, Matsubara, Osaka 580, Japan

Bioactivity-guided separation of a CH_2Cl_2-MeOH extract of Balanites aegyptiaca Del afforded four new cytostatic saponins named balanitin-4 (I), -5 (II), -6 (III) and -7 (IV). On the basis of enzymatic hydrolyses results and glycosidation NMR chemical shifts employing the peracetates, structures I - IV were established as yamogenin 3β-O-β D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside, yamogenin 3β-O-α-L-rhamnopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside, yamogenin 3β-O-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2(]-β-D-glucopyranoside and diosgenin 3β-O-β-D-xylopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)-[α L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside, respectively.

KEYWORDS balanitin; Balanites aegyptiaca; cytotoxicity; saponin; yamogenin glycoside; diosgenin glycoside; P-388 lymphocytic leukemia cell line

Balanites aegyptiaca Del. (Balanitaceae) is widely used in Africa as a component of various primitive medicines used for aborifacient, antiseptic, antiviral (Herpes Zoster), malerial, molluscidal, syphilatic and vermifuge purposes.[2] Recently, the molluscidal steroidal saponins, balanitin-1, -2 and -3, have been isolated from the root and bark of this plant.[3] As part of our evaluation of higher plant antineoplastic and/or cytostatic constituents, we have examined the seed components of this plant using the murine P-388 lymphocytic leukemia (PS system) in cell culture.[4] A 1978 recollection (107 kg) of its seeds produced by B. aegyptiaca were broken and extracted using our CH_2Cl_2-MeOH (followed by dilution with H_2O) technique.[5,6] The aqueous phase was successively partitioned[7] between CH_3OH-H_2O (9:1→3:2) employing hexane→CCl_4→CH_2Cl_2→BuOH. The most encouraging PS activity was located in the BuOH fraction. Bioassay-directed fractionation employing[7] Sephadex LH-20→silica gel column chromatography HPLC afforded four new PS active constituents designated[3] balanitin (Bal)-4, -5, -6, and -7, respectively (Table I). Analysis of the IR,[8] ^1H- and ^{13}C-NMR spectra of the parent balanitins and their acetates suggested that Bal-4 (I), -5 (II) and -6 (III) were glycosides of yamogenin, while Bal-7 (IV) was a glycoside of diosgenin.

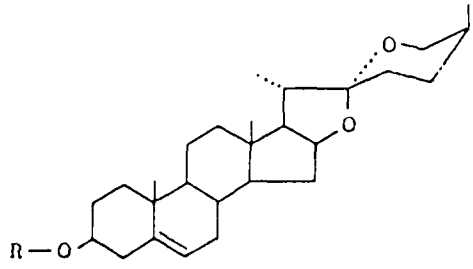
Bal-6 (III): mp 278-280°C (from MeOH), $[\alpha]_D^{20}$ -89° (c = 0.67, pyridine), $C_{45}H_{72}O_{17}$ by FAB-MS m/z: 885 $[M + H]^+$ 907 $[M + Na]$; IR ν_{max}^{KBr} cm^{-1}, 3430, 3280 (OH), 1630 (C=C), 980, 910, 890 [intensity 910 >890, 25(S)-spiroketal],[8] gave on acetylation nonacetate V (the ^1H-NMR spectrum showed the presence of two glucose and one rhamnose unit). The FAB-MS of Bal-6 (III) gave ions at m/z 761 $[M + Na - 146]^+$ and m/z 739$[M + H - 146]^+$ due to the loss of one rhamnose unit, and fragments at m/z 745 $[M + Na - 162]^+$ and 723 $[M + H - 162]^+$ arising from the loss of one glucose unit.[3] Hense both rhamnose and glucose were found linked to glucose in turn attached to yamogenin. In the ^{13}C-NMR spectrum[9] of peracetate V, the C-2 and C-4 signals (δ 76.54 and 77.59 ppm) of one glucose appear shifted downfield by 4.55 and 8.43 ppm, respectively, relative to methyl tetraacetylglucoside. These glycosidation ^{13}C-NMR chemical shifts[10] indicate that the glycosidic linkages exist at the glucose 2- and 4-positions. Enzymatic hydrolysis of trisaccharide III with cellulase followed by acetylation gave hexaacetate VI, where the sugar unit consisted of one glucose and one rhamnose (deduced from the ^1H-NMR spectrum). The proton signal of the glucose peracetate (VI) 2-position was found shifted upfield, relative to methyl

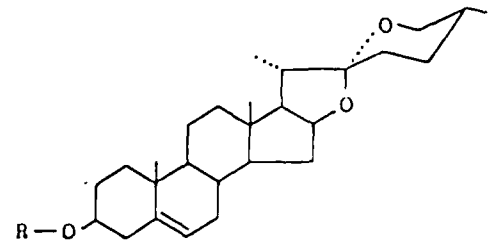
tetraacetylglucoside. In turn this indicated that rhamnose was linked to the 2-position of glucose derivative VI.[11] Based on this evidence, the structure of peracetate V was elucidated and Bal-6 should be represented by structure III.[12]

Bal-4 (1): mp 271-272°C (MeOH), $[\alpha]_D^{20}$ -61° (c = 0.9, pyridine), $C_{51}H_{82}O_{22}$ by FAB-MS m/z, 1047 [M + H]$^+$, 1069 [M + Na]$^+$; IR ν_{max}^{KBr} cm^{-1}, 3380 (OH), 1630 (C=C), 980, 912,>890 [intensity 912 >890, 25(S)-spiroketal],[8] gave on acetylation dodecaacetate VII. The peracetate ^{1}H-NMR spectrum indicated the presence of three glucose and one rhamnose unit. In the peracetate ^{13}C-NMR spectrum the C-2 and C-4 signals (δ 76.46 and 77.24) of one glucose and the C-3 signal (δ 79.77) of another glucose were located shifted downfield by 4.49, 8.09 and 6.19 ppm, respectively. Enzymatic hydrolysis of saponin I with naringinase followed by acetylation afforded Bal-6 peracetate (V) and allowed assignment of structure I to Bal-4.[12]

Table I. P388 cell line Evaluation of the Bal-4-6(I-III) Cell Growth Inhibition

Steroidal Saponin	P-388 (ED_{50} μg/ml)[1a]
I	0.41
II	2.40
III	0.21
IV	0.22
5-Fluorouracil (standard)	8.0×10^{-2}





I R = Glc(2→1)Rha, Glc(4→1)Glc

II R = Glc(2→1)Rha, Glc(4→1)Rha

III R = Glc(2→1)Rha, Glc(4→1)Glc

V R = Glc(Ac)$_2$(2→1)Rha(Ac)$_3$, Glc(Ac)$_4$(4→1)Glc

VI R = Glc(Ac)$_3$(2→1)Rha(Ac)$_3$

VII R = Glc(Ac)$_2$(2→1)Rha(Ac)$_3$, Glc(Ac)$_3$(4→1)Glc(Ac)$_4$

VIII R = Glc(Ac)$_2$(2→1)Rha(Ac)$_3$, Glc(Ac)$_3$(4→1)Rha(Ac)$_3$

IV R = Glc(2→1)Rha, Glc(4→1)Xyl

IX R = Glc(Ac)$_2$(2→1)Rha(Ac)$_3$, Glc(Ac)$_3$(4→1)Xyl(Ac)$_3$

X R = Glc(Ac)$_3$(2→1)Rha(Ac)$_3$

Glc: β-D-glucopyranosyl
Rha: α-L-rhamnopyranosyl
Xyl: β-D-xylopyranosyl

Bal-5 (II): mp 203-207°C (MeOH), $[\alpha]_D^{20}$ -78 (c=1.47, pyridine), $C_{51}H_{82}O_{21}$ by FAB-MS m/z, 1031 $[M + H]^+$, 1053 $[M + Na]^+$; IR ν_{max}^{KBr} cm^{-1}, 3380 (OH), 1640 (C=C), 980, 912, 890 [intensity 912 >890, 25(S)-spiroketal],[8] gave on acetylation undecaacetate VIII and for this derivative the ^{1}H-NMR spectrum indicated the presence of two glucose and two rhamnose units. The ^{13}C-NMR spectrum of peracetate VIII provided useful data. The C-2 and C-4 signals (δ 76.53 and 77.33) of one glucose and the C-3 signals (δ 81.80) of another glucose appeared shifted downfield by 4.54, 8.18 and 8.22 ppm, respectively. Enzymatic hyrolysis of steroidal saponin II with naringinase followed by acetylation gave peracetate V. The sum of this evidence led to structure II for Bal-5.[12]

Bal-7 (IV): mp 273-280°C (MeOH), $[\alpha]_D^{20}$ -83° (c = 0.83, pyridine), $C_{50}H_{80}O_{21}$ by FABS-MS, m/z, 1017 $[M + H]^+$, 1039 $[M + Na]^+$; IR ν_{max}^{KBr} cm^{-1}, 3380 (OH), 1630 (C=C), 980, 915, 895 [intensity 915 <895, 25(R)-spiroketal],[8] gave on acetylation undecaacetate IX. The ^{1}H-NMR spectrum of the peracetate indicated two glucose, one rhamnose, and one xylose unit. Enzymatic takadiastase hydrolysis of IV followed by acetylation provided hexaacetate X. General features of the peracetate ^{1}H-NMR spectrum were identical with those of peracetate VI except that the proton and carbon signals of the aglycone corresponded to those of diosgenin. Therefore, peracetate X was assumed to have a structure, in which the yamogenin aglycone of saponin VI has been replaced by diosgenin. As expected the C-2 and C-4 ^{13}C-NMR signals (δ 76.68 and 77.19) of one glucose and the C-3 signal (δ 80.48) of the second glucose in peracetate IX were respectively shifted downfield by 4.47, 8.04 and 80.48 ppm. The above-summarized evidence allowed assignment of structure IV to Bal-7.[12]

ACKNOWLEDGEMENT

The ASU-CRI group thanks for financial assistance the Arizona Disease Control Research Commission, Eleanor W. Libby, The Waddell Foundation (Donald Ware), the Fannie E. Rippel Foundation, Herbert K. and Dianne Cummings, the Nathan Cummings Foundation, Inc., Lotte Flugel, Polly J. Trautman, Grant CA-30311-01-03 awarded by the National Cancer Institute and Contract NO1-CM-97262 with the Division of Cancer Treatment, NCI, NIH, the National Cooperative Drug Discovery Group Grant No. AI25696-02 and the U. S. Army Medical Research and Development Command under Grant No. DAMD17-89-Z-9021. For other assistance we thank Drs. J. M. Schmidt, and M. I. Suffness, and Messrs. P. J. Daschner and L. Williams. We also thank Professor K. Nakanishi for a specimen of balanitin-1. The Osaka co-authors are very grateful to Dr. H. Tanaka and Mr. N. Shigematsu, Fujisawa Pharmaceutical Co., Ltd., Tsukuba Research Laboratories, for the FAB-MS measurements and to Dr. A. Ito, Sankyo Co., Ltd., for a gift of crude takadiastase.

REFERENCES AND NOTES

1) Part 196 of the series Antineosplastic Agents. For series part 195 refer to: S. B. Singh, G. R. Pettit and D. L. Doubek, J. Nat. Prod. in preparation.
(a), ASU-CRI, (b), Osaku University.

2) J. O. Kokwano, Medicinal Plants of East Africa, P. 34 East Africa Literature Bureau, Kampala, Nairobi, Dar es Salaam (1976); and J. A. Duke, Medicinal Plants of the Bible, Trado-Medic Books, New York, 1983, p 28.

3) H.-W. Liu and K. Nakanishi, Tetrahedron, 38, 513 (1982).

4) R. I. Geran, N. H. Greenberg, M. M. McDonald, A. M. Schumacher and B. J. Abbott, Cancer Chemother. Rep., (Part 3) 3, I (1972).

5) G. R. Pettit, Y. Fujii, J. A. Hasler, J. M. Schmidt and C. Michel, J. Nat. Prod., 45, 263 (1982).

6) G. R. Pettit, V. Gaddamidi, A. Goawami and G. M. Cragg, 47, 796 (1984).

7) G. R. Pettit, Y. Kamano, R. Aoyagi, C. L. Herald, D. L. Doubek, J. M. Schmidt and J. J. Rudloe, Tetrahedron, 41, 985 (1985).

8) T. Konishi and J. Shoji, Chem. Pharm. Bull., 27, 3086 (1979).

9) Assignmerts of the ^{13}C-NMR signals in the sugar moiety of the peracetates (V and VII - IX) were based on a correlation with the fully assigned proton signals in the ^{1}H-^{13}C heteronuclear shift correlated (HETCOR) 2D-NMR spectrum. The ^{1}H-NMR signals in the sugar moiety were assigned on the

basis of ^{1}H-^{1}H homonuclear decoupling.

10) K. Tori, S. Seto, Y. Toshimura, M. Nakamura, Y. Tomita and H. Ishii, Tetrahedron Lett., 4167 (1976).

11) An upfield shift of proton signals corresponding to the glycosidic linkage was observed in the NMR spectra of acetates V and VII-IX.

12) The anomeric configurations of glucose and xylose were deduced from the J_{1H-1H} and J_{13C-1H} values[13] given by the peracetate anomeric positions and that of rhamnose was arrived at from the anomeric proton chemical shifts displayed by the glycosides and the J_{13C-1H} value for the peracetate anomeric carbons.

13) K. Bock, I. Lundt and C. Pedersen, Tetrahedron Lett., 1037 (1973).

J. Nat. Prod.
2/28/89

ANTINEOPLASTIC AGENTS. 107. ISOLATION OF ACTEOSIDE AND ISOACTEOSIDE FROM CASTILLEJA LINARIAEFOLIA (SCROPHULARIACEAE)[1]

GEORGE R. PETTIT*, ATSUSHI NUMATA,[2] TSURUKO TAKEMURA,[2] RICHARD H. ODE, A. S. NARULA, JEAN M. SCHMIDT, GORDON M. CRAGG, AND CHARLES P. PASE

Cancer Research Institute and Department of Chemistry
Arizona State University, Tempe, Arizona 85287

---

[1]The previous report in this series corresponds to Ref. 1

[2]Osaka College of Pharmacy, Osaka 580, Japan

*Author to whom correspondence should be addressed

INTRODUCTION

In mountainous areas of Northern Arizona (2) and Southern Utah, Castilleja linariaefolia Benth is known as an Indian paint-brush. While some fifty species of the large (3000 species and 220 genera) Scrophulariaceae family have been used in primitive cancer treatment only one represented the Castilleja genus (3), namely, the Mexican (Yucatan) Castilleja communis Benth (4).

In 1966 extracts of Castilleja linariaefolia, collected north of Arizona's Grand Canyon, gave confirmed activity against the U. S. National Cancer Institute's Walker carcinosarcoma 256 (intramuscular WM system) in the rat. Each of the major plant parts appeared to contain the anticancer constituent(s) with the flowers indicating the greatest concentration and causing a 90% inhibition of tumor growth at 266 mg/kg. The stem components appeared next most active and led to a 58% inhibition of tumor growth at 400 mg/kg. As the root material was less promising with 25% inhibition (400 mg/kg), a chemical investigation of C. linariaefolia was begun (in 1967) primarily with the stems and flowers using the WM system as the bioassay guide. When the murine P388 lymphocytic leukemia (PS system) became available for this research in 1971 and a key fraction showed T/C 125-165% (10-40 mg/kg), the WM system was discontinued as a guide to fractionation. However, somewhat erratic results were obtained with both in vivo systems, and this served to complicate the isolation studies with this heretofore chemically unexplored species.

A number of increasingly larger recollections of C. linariaefolia were required over the 1967-79 period as improved biological (the PS cell line) and chemical techniques were applied to this problem. Two

of the PS cell growth inhibitory[3] constituents were eventually found to be the known glycosides acteoside (6-9) and isoacteoside (8). Both glycosides[4] have not hitherto been found in a plant of the Scrophulariaceae and were isolated utilizing a large scale (~500 kg) 1978 recollection of the paint-brush from Southern Utah. The research was completed using a series of recently developed (Cf. 10) experimental procedures augmented by droplet countercurrent chromatography (dccc).[5] As part of this study D-mannitol was also isolated.

The structure of acteoside (1a) was first assigned on the basis of detailed spectral analysis (uv, ir, etc.) and identification of alkaline and acid hydrolysis products. Later the structure was confirmed by comparison with an authentic sample.[6] The general spectral features of isoacteoside closely resembled those of acteoside, except that the L-rhamnose unit methyl group signal in the ^{1}H-nmr spectrum was shifted from δ 1.12 to 1.27 ppm. Also the C-6

---

[3]The PS *in vitro* studies were conducted in our laboratory according to procedures developed by the National Cancer Institute, and PS *in vivo* bioassays were performed under auspices of the NCI (5).

[4]These caffeoyl glycosides have been isolated from a Labiatae species (8) and acteoside also occurs in a Gesneriaceae (7) and two Oleaceae (6, 9).

[5]The dccc technique has previously been used in the separation of iridoid glycosides from Castilleja miniata (11). Interestingly, dccc was the only effective method found for separation of myricoside, a bioactive substance closely resembling acteoside in structure (12).

[6]An authentic sample of acteoside was kindly provided by Professor I. Nishioka, Faculty of Pharmaceutical Sciences, Kyushu University.

and C-3 D-glucose unit carbon signals in the ^{13}C-nmr spectrum were shifted from δ 62.43 to 64.70 and 81.64 to 84.15 ppm, respectively. These data suggested an isomeric relationship, and this was confirmed by a series of methylation, acetylation and hydrolysis experiments (11, 13, 14).

Both acteoside and isoacteoside exhibited in vitro cell growth inhibitory activity (9PS; ED_{50} 2.6 and 10 µg/ml respectively). An appropriate in vivo antineoplastic evaluation will require additional amounts of both acteoside and isoacetoside. Because of the antibacterial (12) and cAMP phosphodiesterase inhibitory activity shown by several closely related natural products these glycosides should undergo further biological study. Meanwhile, we are pursuing additional antineoplastic constituents of C. linariaefolia.

EXPERIMENTAL

All melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Infrared spectra were recorded with a Hitachi EPI-G2 spectrometer. The ^{1}H-nmr spectra were measured with a Hitachi R 40 spectrometer at 90 MHz and the ^{13}C-nmr spectra with a JEOL JNM FX-200 spectrometer at 50.3 MHz. Chemical shifts are given in parts per million (δ) downfield from tetramethylsilane as an internal standard. Solution phase sims (15) mass spectra were obtained using a Varian MAT 312 spectrometer equipped with a modified capillaritron source (15) and 0.14 M sodium iodide in sulfolane as liquid phase. The ei mass spectra were recorded using a Hitachi M-70 spectrometer.

PLANT MATERIAL.-A final recollection of Castilleja linariaefolia, Benth (~500 kg dry wt) by GRP, D. L. Herald, D. L. Doubek, D. Takata and T. E. Rawson, was accomplished August 1978 in the Dixie National Forest, Garfield Co., Utah. The collections were made at 7000-8000 ft elevation and identified by one of us (CPP) in the USDA Laboratory and by E. Lehto in the Dept. of Botany at Arizona State University. Herbarium specimens are maintained in the Dept. of Botany and Cancer Research Institute at Arizona State University.

PLANT EXTRACTION.-Dried plant material (36 kg; leaves, stems and roots) from the 1978 recollection was extracted with a mixture (160ℓ) of methylene chloride and methanol (1:1, 13) at ambient temperature for three weeks. The extract was separated into methylene chloride and aqueous phases on addition of 25% by volume of water. The aqueous phase was adjusted by addition of methanol and methylene chloride in the ratio aqueous phase-methanol-methylene chloride (4:1:2), and the

plant was extracted with this mixture for seven weeks. Addition of 15% by volume of water separated the methylene chloride phase which was combined with that obtained from the first partition. Concentration gave a PS *in vivo* inactive methylene chloride extract (712 g; PS, ED_{50} 19 μg/ml; 3PS, inactive at 12.5-100 mg/kg). The aqueous phase was concentrated to give a marginally *in vivo* active extract (3.5 kg; 9PS, ED_{50} >100 μg/ml; 3PS, T/C 120% at 25 mg/kg).

SOLVENT PARTITION SEQUENCE.-A portion of the aqueous extract (180 g) was successively partitioned between methanol-water 9:1 (1800 ml), 4:1 and 1:1 with hexane (3 x 1800 ml), carbon tetrachloride (3 x 1800 ml) and methylene chloride (3 x 1800 ml), respectively. Concentration of the partition fractions gave hexane (1.5 g; 9PS, ED_{50} 6 μg/ml), carbon tetrachloride (1.2 g; 9PS, ED_{50} 24 μg/ml), methylene chloride (4.6 g; 9PS, ED_{50} 51 μg/ml), and aqueous (142.8 g; 9PS, ED_{50} 36 μg/ml) fractions. None of these fractions exhibited 3PS *in vivo* activity when tested at dose levels of 3.12-25 mg/kg. However, in a number of earlier experiments, e.g., employing a 1972 recollection, fractions were obtained at this stage with PS T/C 165% at 40 mg/kg.

ISOLATION OF ACTEOSIDE (1a) AND ISOACTEOSIDE (1b).-An aliquot (10.22 g) of the aqueous partition fraction was chromatographed on Sephadex LH-20 (805 g; 77 x 8 cm) using methanol-water (4:1) as eluent. Fractions were monitored by ppc. After elution of 5.1 g of material, a fraction (2 g) was obtained from which D-mannitol (0.252 g) was isolated as colorless needles, mp 171-172°, and found identical (ir, tlc) with an authentic sample. Further elution gave 1.8 g of inactive material followed by a 9PS *in vitro* active (ED_{50} 4.1 μg/ml; 3PS, inactive at 3.12-25 mg/kg) fraction (1.3 g). A 1.94 g portion of this

fraction (obtained on repetition of the Sephadex LH-20 chromatographic step) was treated with methanol. The soluble fraction (1.38 g) was dissolved in a minimum volume of the upper layer prepared from chloroform-methanol-water (5:5.7:3) and placed in the transfer tube of a dccc apparatus filled with the upper layer of the same solvent system as stationary phase. The flow rate of the moving lower phase was 0.66 ml/min. Three fractions were collected on the basis of monitoring by PPC. Acteoside (1a, 0.388 g) was isolated as a pale yellow amorphous powder from the third fraction. The first fraction (0.174 g) was again subjected to dccc to give isoacteoside (1b, 0.107 g) as a light brown amorphous powder. Acteoside (1a) exhibited the following physical properties: mp 145-149° (lit. 6, mp 147-150°); and solution phase SIMS m/z 625 ($[M+H]^+$; 21%), 480(10%), 472(24%), 325 ($[M+H\text{-caffeoyl-3,4-dihydroxyphenethyl}]^+$ 100%). The identity was confirmed by direct comparison of ^1H- and ^{13}C-nmr spectra with an authentic sample.[6] Treatment of acteoside (1a) with acetic anhydride-pyridine followed by silica gel column chromatography gave the peracetate as a colorless amorphous powder: UV (CH_3OH)λ_{max} 283 (log ε 4.19) nm; the CD spectrum was identical with that already published (12). Isoacteoside (1b) exhibited mp 136-139°; and solution phase SIMS m/z 625 ($[M+H]^+$; 22%), 480 (29%), 472 (30%), 325($[M+H\text{-caffeoyl-3,4-dihydroxyphenethyl}]^+$ 100%). Acetylation and chromatography of the product as described for acteoside gave the peracetate as a colorless amorphous powder: UV (CH_3OH)λ_{max} 280 (log ε 4.53) nm.

ACKNOWLEDGMENTS

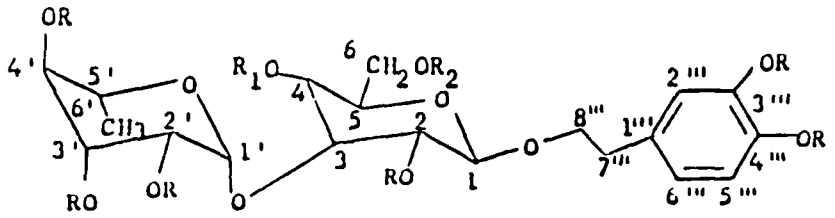
Financial support for this investigation was provided by Eleanor W. Libby, the Waddell Foundation (David Ware), Mary Dell Pritzlaff, the Olin Foundation (Spencer T. and Ann W.), the Fannie E. Rippel Foundation, the Flinn Foundation, the Robert B. Dalton Endowment Fund, Virginia L. Bayless, Southwest Forest Industries, Elias M. Romley, NIH Grant CA 30311-01-03, Contract NO1-CM-97297 with the Division of Cancer Treatment, NCI, DHHS, the National Cooperative Drug Discovery Group Grant No. AI 25696-02 and the U.S. Army Medical Research and Development Command under Grant No. DAMD17-89-Z-9021. We also appreciate other assistance contributed by Drs. J. J. Einck, W. C. Fleming, C. L. Herald, P. Lohavanijaya, M. L. Suffness, and I. Nishioka, as well as G. C. Bryan and J. F. Day, M. J., W. E., M. S., and G. R. (III) Pettit and those already named as contributing to the extensive plant collections and recollections noted in the experimental section.

LITERATURE CITED

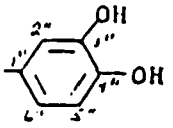
1. L. R. Nassimbeni, M. L. Niven, G. M. Cragg, and G. R. Pettit, Acta. Cryst., submitted.
2. T. H. Kearney and R. H. Peebles and collaborators, "Arizona Flora", University of California Press, Berkeley and Los Angeles, Calif., 1951, p. 789.
3. J. L. Hartwell, Lloydia, 34, 204 (1971).
4. P. C. Stanley, "Flora of Yucatan", Publ. Field Museum Nat. Hist. No. 279, Bot. Ser. 3(3), 157 (1930).
5. R. I. Geran, N. H. Greenberg, M. M. MacDonald, A. M. Schumacher, and B. J. Abbott, Cancer Chemother, Rep., Part 3, 3, 1 (1972).
6. L. Birkofer, C. Kaiser, and U. Thomas, Z. Naturforsch., 23b, 1051 (1968).
7. G. Nonaka and I. Nishioka, Phytochemistry, 16, 1265 (1977).
8. T. Miyase, A. Koizumi, A. Ueno, T. Noro, M. Kuroyanagi, S. Fukushima, Y. Akiyama, and T. Takemoto, Chem. Pharm. Bull., 30, 2732 (1982).
9. S. Kitagawa, H. Tsukamoto, S. Hisada, and S. Nishibe, Chem. Pharm. Bull., 32, 1209 (1984).
10. G. R. Pettit, Y. Kamano, R. Aoyagi, C. L. Herald, D. L. Doubek, J. M. Schmidt, and J. J. Rudloe, Tetrahedron, 41, 985 (1985).
11. K. Hostettmann, M. Hostettmann-Kaldas, and O. Sticher, J. Chromatog., 186, 529 (1979).
12. R. Cooper, P. H. Solomon, I. Kubo, K. Nakanishi, J. N. Shoolery, and J. L. Occolowitz, J. Am. Chem. Soc., 102, 7953 (1980).
13. J. Chopin, B. Roux, M. L. Bouillant, A. Durix, A. D'Arcy, T. Mabry, and H. Yoshioka, Compt., Rendus, 268, 980 (1969).

14. G. R. Pettit, G. M. Cragg, and M. Suffness, J. Org. Chem., 50, 5060 (1985).

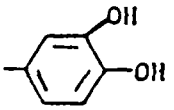
15. G. R. Pettit, C. W. Holzapfel, G. M. Cragg, C. L. Herald, and P. Williams, J. Nat. Prod., 46, 917 (1983).



1a, R=R$_2$=H, R$_1$=COCH=CH



b, R=R$_1$=H, R$_2$=COCH=CH



ABSTRACT

The Southwestern (USA) paint-brush, Castilleja linariaefolia Benth., yielded extracts that displayed confirmed in vivo activity against the National Cancer Institute's murine P388 lymphocytic leukemia (PS system). Separation employing droplet countercurrent chromatography as one of the key techniques, guided by cell line inhibition results from the PS leukemia system, led to isolation of two cell growth inhibitors found to be acteoside (**1a**, ED_{50} 2.6 μg/ml) and isoacteoside (**1b**, ED_{50} 10 μg/ml). The structures of both glycosides were established by detailed spectral measurements and degradation studies. Mannitol was also found in this plant.

JNP-
Note
5/3/89

ANTINEOPLASTIC AGENTS 162. *ZEPHYRANTHES CANDIDA*[1]

George R. Pettit,* Gordon M. Cragg, Sheo Bux Singh,

James A. Duke and Dennis L. Doubek

Cancer Research Institute and Department of Chemistry

Arizona State University, Tempe, Arizona 85287-1604

Received

*Author to whom correspondence should be addressed.

[1]Refer to Ref. 1 for the preceding contribution.

Amaryllidaceous plants such as Narcissus poeticus were recorded in the Bible as well established treatments for cancer (2) and others were in use by the Greek physicians of 4 BC (3). The first isolation, in 1877 (4), of a biologically active Amaryllidaceae constituent, the now well-known lycorine (5), was an early achievement of organic chemistry and such studies have been intensifying (cf,5, 6-8). In 1984 we reported discovery and structural elucidation of a strongly antineoplastic phenanthridone designated pancratistatin (1) produced by species of the genera Pancratium littorale, (3,9) and Zephyranthes grandiflora (6).

In 1964, extracts of the medicinal (6) Zephyranthes candida L. (obtained in Hong Kong) had already proved active (KB cell line)[2] in the U. S. National Cancer Institute's exploratory research program, but we were unable to obtain a recollection (People's Republic of China) until 1982. Earlier (1955) Boit and Ehmke (10) isolated four alkaloids from the Dutch Z. Candida representing the pyrrolo [de] phenanthridine (lycorine), pretazettine (tazettine), and 5, 10b-ethanophenanthridine (haemonthidine and nerinine) ring systems. The study was extended in 1964-65 (11,12) to isolation of the dihydrolycorine and zephyranthine from a Japanese variety and in 1978 to a flavone glycoside (13). We now have found that the principal antineoplastic (murine P388 lymphocytic leukemia, PS system, 13) constituent of Z. candida to be trans-dihydronarciclasine (1b) previously (14) prepared[3] by hydrogenation of narciclasine (2a) and heretofore unknown as a biosynthetic product.

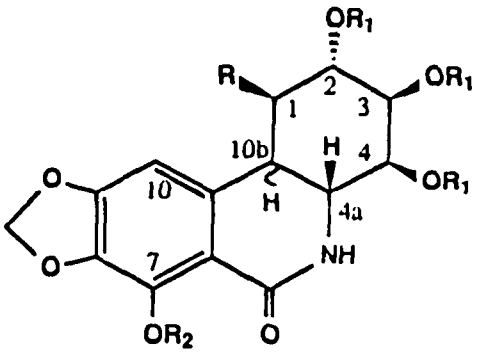
---

[2]Cell line from a human epidermoid carcinoma of the nasopharynx.

[3]We thank Professor A. Mondon for samples of trans-dihydro-nacriclasine and its peracetate as well as the corresponding cis-dihydro-compounds (see ref. 14).

Ground bulbs of Z. candida were extracted with methylene chloride-methanol (1:1) at ambient temperature. After addition of water, the aqueous phase was concentrated and extracted with n-butanol. The PS active (cell line) butanol extract was concentrated and triturated with methanol to provide a fraction that was separated (guided by PS bioassay) by successive Sephadex LH-20 and silica gel column chromatographic steps. The resulting enriched active (ED_{50} 0.0034 ug/ml) fraction was acetylated and separated on a column of silica gel to yield trans-dihydronarciclasine peracetate (1c), PS (ED_{50} 3.2×10^{-3} µg/ml) as the major component. The structure of the peracetate was established by detailed spectral analysis (e.g., see 3) and comparison with an authentic sample as well as with the product obtained by catalytic hydrogenation (Adam's catalyst in acetic acid at 50 psi) of narciclasine, followed by acetylation. Hydrogenation afforded as major product the expected cis-dihydronarciclasine accompanied by the trans-isomer. Facile deacetylation of the phenolic acetoxy group was observed during chromatography and in methanolic solutions to give the 7-hydroxy-2,3,4-triacetoxy derivative **1d**. Trans-dihydronarciclasine (prepared from the acetate) was found to strongly inhibit the PS leukemia with ED_{50} 0.0032 µg/ml, while the synthetic cis-dihydro analog (**1e**) led to PS ED_{50} 0.024 µg/ml.

Isolation of trans-dihydronarciclasine (**1b**) as the major antineoplastic constituent of Z. candida has revealed another interesting and potentially useful amaryllidaceae biosynthetic product. Doubtlessly, further study of this very productive plant family for anticancer and other medically useful components will prove rewarding and is in progress.



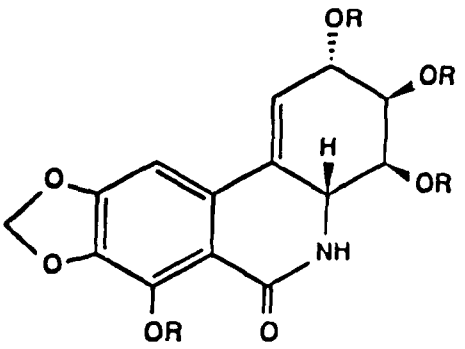
1a. R = OH, $R_1 = R_2 = H$, 10b = α-H

b. $R = R_1 = R_2 = H$, 10b = α-H

c. R = H, $R_1 = R_2 = COCH_3$, 10b = α-H

d. $R = R_2 = H$, $R_1 = COCH_3$, 10b = α-H

e. $R = R_1 = R_2 = H$, 10b = β-H



2a. R = H

b. $R = COCH_3$

EXPERIMENTAL

General Methods.-Details of general procedures and chromatographic techniques were provided in our earlier summaries (3,6).

Plant Material.-Zephyranthes candida (Lindl.) Herb. was recollected in China in 1981 (received February 1982) as part of the NCI-USDA collaborative program directed by Drs. J. L. Hartwell and M. Suffness.

Extraction.-Freshly ground bulbs (18 kg) were stored in methanol-methylene chloride (1:1, 32 liters) for 10 days. Addition of water (15% by volume) caused separation of the methylene chloride phase. To the aqueous phase was added methanol and methylene chloride to increase the original total volume by 50 and 25% respectively. The plant was extracted with this mixture for a further 80 days. Addition of water (25% by volume) allowed the methylene chloride phase to separate which was combined with the first methylene chloride extract and concentrated to a 109 g residue (PS ED_{50} 3.5 µg/ml). The aqueous phase was concentrated and partitioned between water (6 liters) and n-butanol (4x6 liters). Concentration of the butanol extract to a small volume and addition of methanol (2 liters) gave an active methanol-soluble fraction (149 g; PS ED_{50} 0.27 µg/ml). Upon further dilution with methanol (600 ml) and methylene chloride (400 ml) the solution was filtered to yield 28 g of solid (PS ED_{50} 1.6 µg/ml). The filtrate was chromatographed on a column of Sephadex LH-20 (2.5 kg) using methanol-methylene chloride (3:2) as eluent.

Isolation of Trans--dihydronarciclasine.-Elution (the preceding LH-20 column) between volumes 7215-16950 ml gave a 6.2g fraction (PS $ED_{50} < 0.02$ ug/ml). Trituration with acetone (50 ml) provided a light orange solid (2.72 g PS ED_{50} 0.016 µg/ml) and a soluble fraction (3.5 g; PS ED_{50} 0.0043 µg/ml). When the orange solid was triturated with methanol-methylene chloride (1:1; 3

x 10 ml, 1 day), followed by methanol (5 ml; 2 days) a soluble fraction (2.58 g) was obtained similar (by tlc) to the acetone-soluble fraction. An aliquot of the acetone-soluble fraction (1.76 g) and the latter soluble fraction (2.58 g) were combined and the mixture subjected to rapid chromatography on a column of silica gel (200 g). Gradient elution with methylene chloride (1 liter) to 99:1 95:5 methylene chloride-methanol followed by 9:1 (2 liters) gave a fraction (1.05 g) which was triturated with methanol (5 ml; 1 day) to give a buff-colored solid (0.20 g; PS ED_{50} 0.0034 μg/ml). Half of the solid was acetylated (6 ml of 1:1 acetic anhydride-pyridine, 24 h room temperature) and the product (0.12 g) was chromatographed on a column of silica gel (Lobar B column). Development with methylene chloride (200 ml) and 99:1 methylene chloride-methanol (400 ml), followed by 49:1 afforded (between 675 and 725 ml, total eluant volume) trans-dihydronarciclasine-2,3,4-triacetate (16 mg) which recrystallized from methanol-methylene chloride as small colorless needles: mp 309-311° [lit (14) mp 293°]; $[\alpha]^{32}_{D}$ + 81.94° (c, 0.72, $CHCl_3$); UV λ_{max} MeOH (log ε) 231 (4.04), 239 (4.01), 280 (3.75), 310 (3.33) nm; ^{1}H-nmr (400 MHz, $CDCl_3$) 1.914 (1H, ddd, J=14.0, 12.5, 3.0 Hz, H-1β), 2.086 (6H, s, 2xAc), 2.137 (3H, s, Ac), 2.432 (1H, ddd, J=14.0, 3.5, 3.2 H_z, H-1α), 3.134 (1H,ddd, J=12.7, 12.5, 3.5 Hz, H-10b), 3.777 (1H, dd, J=12.7, 11.8 Hz, H-4a), 5.175 (1H, dd, J=11.8, 3.0 Hz, H-4), 5.189 (1H, m, H-3), 5.438 (1H, dd, J=3.2, 3.0 Hz), 5.855 (1H, s, NH), 6.037, 6.049 (1H each, d, J=1.2 Hz, OCH_2O), 6.323 (1H, s, H-10), 9.704 (1H, s, ArOH). Acetylation (1:1 acetic anhydride-pyridine) led to trans-dihydronarciclasine peracetate identified by tlc and infrared spectral (in chloroform) with an authentic specimen.

Continued elution between volumes 725-760 ml gave a mixture (14 mg) of the above triacetate and trans-dihydronarciclasine peracetate and between volumes 760-810 ml trans-dihydronarciclasine peracetate (80 mg).

Recrystallization from methanol-methylene chloride afforded a pure specimen as colorless needles: mp 181-182° [lit (14), 188-189°]; $[\alpha]^{31}_{D}$ + 123.9° (c, 1.13, $CHCl_3$) [lit (14) $[\alpha]^{20}_{D}$ +128.5° (c, 0.82, $CHCl_3$)]; UV λ_{max} MeOH (log ε) 231 (4.10), 239 (4.09), 280 (3.82), 310 (3.40 nm; IR (KBr) ν_{max} 3600, 3500, 3330 (sh), 3310, 1760, 1730 (sh), 1670, 1634, 1505, 1487, 1460, 1371, 1345, 1298, 1255, 1235, 1172, 1080, 1051, 1031, 930 cm^{-1}; ^{1}H-nmr (400 MHz, $CDCl_3$) 1.906 (1H, ddd, J=14.0, 12.7, 3.0 Hz, H-1β), 2.054, 2.071, 2.139 (3H each, $COCH_3$), 2.364 (3H, $ArOCOCH_3$), 2.428 (1H, ddd, J=14.0, 3.5, 3.2 Hz, H-1α), 3.140 (1H, ddd, J=12.7, 12.0, 3.5 Hz, H-10b), 3.762 (1H, dd, J=12.0, 10.8 Hz, H-4a), 5.156 (1H, dd, J=10.8, 3.0 Hz, H-4), 5.192 (1H, m, H-3), 5.416 (1H, dd, J=3.2, 3.0 Hz, H-2), 5.810 (1H, s, NH), 6.065, 6.073 (1H each, d, J=1.2 Hz, $-O-CH_2-O$), 6.642 (1H, s, H-10) and ^{13}C-nmr (22.63 MHz, $CDCl_3$) 170.34, 169.36, 169.14 (4C, 4x$O\underline{C}OCH_3$), 163.35 (C-6), 152.40 (C-9), 139.63 (C-7), 137.00 (C-10a), 134.33 (C-8), 116.04 (C-6a), 102.91 (OCH_2O), 102.00 (C-10), 71.62, 68.60, 67.43 (3x$\underline{C}HOCOCH_3$), 52.41 (C-4a), 35.61 (C-10b) 27.00 (C-1), 21.02, 20.86, 20.70 (4c, 4x$OCO\underline{C}H_3$) ppm. Hreims (m/z) 477.1258 (m^+, 3.09%, calcd 477.1271 for $C_{22}H_{23}NO_{11}$) and 435.1165 (100%, calcd 435.1166) for $C_{20}H_{21}NO_{10}$.

ACKNOWLEDGMENTS

We are very appreciative of financial assistance provided by the Arizona Disease Control Research Commission, Eleanor W. Libby, The Waddell Foundation (Donald Ware), the Fannie E. Rippel Foundation, Herbert K. and Dianne Cummings, (the Nathan Cummings Foundation, Inc.), Virginia Piper, Lotte Flugel, Polly J. Trautman, Grant CA-30311-01-03 awarded by the National Cancer Institute and Contract NO1-CM-97262 with the Division of Cancer Treatment, NCI, NIH, and the U. S. Army Medical Research and Development Command under Grant No. DAM17-89-Z-9021. For other assistance we thank Drs. J. M. Schmidt, V. Gaddamidi, M. I. Suffness, and Messrs P. J. Daschner and L. Williams.

LITERATURE CITED

1. P. M. Blumberg and G. R. Pettit in BBA Reviews on Cancer, in preparation.
2. J. L. Hartwell, Lloydia, **30**, 379 (1967).
3. G. R. Pettit, V. Gaddamidi, D. L. Herald, S. B. Singh, G. M. Cragg, J. Schmidt,F. E. Boettner, M. Williams and Y.Sagawa, J. Nat. Prod., **49**, 995 (1986).
4. J. W. Cook and J. D. Loudon, "The Alkaloids," Ed. by R.H.F. Manske and H. L. Holmes, Academic Press, (1952) Vol. II, Ch. 11, p.331.
5. S. Ghosal, K. S. Saini and S. Razdan, Phytochemistry, **24**, 2141 (1985).
6. G. R. Pettit, V. Gaddamidi, and G. M. Cragg, J. Nat. Prod., **47**, 1018 (1984).
7. M. Kihara, T. Koike, Y. Imakura, K. Kida, T. Shingu, and S. Kobayashi, Chem. Pharm. Bull., **35** 1070 (1987).
8. H.-Y. Li, G.-E. Ma, Y. Xu. and S.-H. Hong, Planta Medica, No. 3, 259 (1987).
9. G. R. Pettit, V. Gaddamidi, G. M. Cragg, D.L. Herald, and Y. Sagawa, J. Chem. Soc., Chem. Commun., 1693 (1984).
10. H. G. Boit and H. Ehmke, Chem. Ber., **88**, 1590 (1955).
11. S. Ozeki, Yakugaku Zasshi, **84**, (12) 1194 (1964).
12. S. Ozeki, Yakugaku Zasshi, **85** (3) 200 (1965).
13. R. I. Green, N. H. Greenberg, M. M. MacDonald, A. M. Schumacher, and B. J. Abbott, Cancer Chemother. Rep., **3**, 1 (1972).; M. Nakayama, T. Horie, M. Tsukayama, M. Masumura, S. Hayashi, Z. Naturforsch Sect. C. Biosci., **33** (7-8), 587 (1978).
14. A. Mondon and K. Krohn, Chem. Ber., **108**, 445 (1975).

ABSTRACT

The Chinese medicinal plant, Zephyranthes candida L. (Amaryllidaceae) was found to contain a cytostatic constituent. Separation of a n-butanol extract directed by results of a bioassay employing the P388 lymphocytic leukemia led to trans-dihydronarciclasine (1b) as the principal cytostatic agent with ED_{50} 3.2×10^{-3} μg/ml.

Can. J. Chem.
6/26/89

ANTINEOPLASTIC AGENTS 168
ISOLATION AND STRUCTURE OF AXINOHYDANTOIN[1]

George R. Pettit*, Cherry L. Herald, Rajesh Gupta, John E. Leet, Daniel E. Schaufelberger, Robert B. Bates[2], Paul J. Clewlow, Dennis L. Doubek, Kirk P. Manfredi, Klaus Rützler[3], Jean M. Schmidt, Franklin B. Ward, Michael Bruck[2] and Fernando Camou[2]

Cancer Research Institute & Department of Chemistry
Arizona State University, Tempe, Arizona 85287

Received

---

*To whom correspondence should be addressed

[1]For Contribution 167 refer to Ref. 1

[2]Department of Chemistry, University of Arizona, Tucson, Arizona 85721.

[3]National Museum of Natural History, Smithsonian Institution, Washington, D.C. 20560.

ABSTRACT

Western (Palau) and Eastern (State of Truk) Caroline Islands and Papua New Guinea sponges of the genera Axinella and Hymeniacidon were found to contain the cytostatic (PS ED_{50} 2.5 and 2.0 μg/ml) and antineoplastic (PS T/C 143 at 3.6 mg/kg and T/C 138 at 3.6 mg/kg) pyrrologuanidines 1a and 1b. The related hydantoin 2, designated axinohydantoin, was also isolated from an Axinella sp. and its structure was assigned by x-ray crystallographic techniques. Present experience with sponges in the Axinella and Hymeniacidon genera suggests that the previously known hymenialdisine (1b) and analogous imidazole derivatives may be widely distributed among these and related orange colored Porifera.

Key Words

Axinohydantoin
hymenialdisine
Axinella
Hymeniacidon
Cytostatic

INTRODUCTION

Early (2) in our evaluation of marine animals as new sources of potentially useful anticancer drugs, good leads were uncovered among the Porifera and this initial (1966-68) promise is now being amply realized (3,4). In 1979 in Palau we collected a Hymeniacidon species (at -40m) and an Axinella sp. that provided extracts with confirmed levels of activity against the U. S. National Cancer Institute's (NCI) murine P388 lymphocytic leukemia (PS system). Other PS active sponge collections were completed in 1981 (Papua New Guinea) and 1985 (Truk, Federated States of Micronesia) that included Axinella carteri (Dendy) and a Hymeniacidon species.

Initial extracts of each sponge were found to provide a confirmed level of activity against the PS system. By means of PS (in vitro) bioassay guided separation procedures, these sponge species led to two cytostatic and antineoplastic alkaloids (1a,b) accompanied in the case of Axinella sp. by a closely related, but marginally (PS ED_{50} 18 μg/ml) inactive component (2). The PS active marine alkaloids proved to be identical[4] with the known (5,6,7) hymenialdisine (1b, ref. 6, PS ED_{50} 2.0 μg/ml and T/C 138 at 3.6 mg/kg)[5] and its debromo-derivative 1a (Ref. 5,6,7 PS ED_{50} 2.5 μg/ml and T/C 143 at 3.6 mg/kg).

The unequivocal x-ray crystal structure of hymenialdisine was nicely established by Cimino and colleagues (5) and reconfirmed in the following year by the Kitagawa group (6). In turn these advances simplified

---

[4]By comparison with authentic specimens provided by Dr. I. Kitagawa (see Ref. 6).

[5]Interestingly, hymenialdisine was previously active in the KB cell line, but inactive employing the P388 leukemia: Cf. Ref. 5. Perhaps the initial negative results were due to the sparingly soluble properties of this pyrrologuanidine.

characterization of the companion substance from Axinella sp., herein named axinohydantoin (2) as a closely related compound. But establishing the exact geometrical configuration for its hydantoin-lactam sp^2 bond required the following crystal structure determination.

Axinohydantoin (2) crystallized as yellow prisms from MeOH that corresponded to $C_{11}H_9BrN_4O_3$ (by hreims) and with one mole of MeOH by cell (space group C2/c) density measurements. A total of 2446 reflections were found usable for crystal structure determination. The problem was solved using MULTAN(8) and refined to R = 0.054 using anisotropic temperature factors for Br and oxygens other than O-3, isotropic temperature factors for the other non-hydrogens, and hydrogens (unrefined) in calculated positions. The HN-1, HN-11, and HN-9 hydrogen bonds were calculated to be 0.95 Å along a line to the respective oxygen. These positions differed very little from those calculated assuming bonding to trigonal atoms. The HN-4 has been shown at the calculated position assuming a trigonal N-4 which is too far (2.4 Å) from the closest O-10 for significant hydrogen bonding; it may actually bend somewhat toward this O-10.

The structure deduced for axinohydantoin (2, Fig 1) was found to be closely related to that of hymenialdisine (1b) with reversal of configuration at the C7-C8 double bond being the most interesting difference. In turn, this suggested that axinohydantoin was not simply a hydrolysis product of guanidine 1b. The most prominent bond length difference between the two structures occurs at C10-O10 with 1.23 Å in hydantoin 2 compared to 1.33 Å for C11-N11 in 1b. No significant differences in bond angles were observed. An angle of 36° was observed between the least squares planes of the two nearly planar five-membered rings in hydantoin 2, compared to 43.8° in guanidine 1b. In both cases, the seven-membered ring has adopted a boat conformation with C-

5 at the prow, and similar torsion angles except for C2-C3-N4-C5 expanding from -10.5° in 1b to -15° in 2, and C2-C13-C7-C6 contracting from 41.1° in 1b to 31° in 2. The twist angle C13-C7-C8-C12 about the carbon-carbon double bond increases from 0.5° in 1b to 10° in 2, presumably to relieve the steric interaction between O-12 and HC-14.

The arrangement of intermolecular hydrogen bonds governing the packing in hydantoin 2 (See Supplementary Material) was found to be completely different than that in guanidine 1b (6). The hydantoin ring in each molecule was found linked to the hydantoin rings of two other molecules *via* base-pairing interactions across centers of symmetry. The pyrrole NH proved to hydrogen bonded to the MeOH solvate oxygen and in turn to O-3. Only a few substances (9-12) with a hydantoin system have been isolated from sponges, and one of these, midpacamide, found by Scheuer and colleagues (9) in an unidentified Marshall Island sponge may be biogenetically related to axinohydantoin. From evidence now in hand, pyrroles 1 and 2 and related substances may prove to be ubiquitous Porifera biosynthetic products.

EXPERIMENTAL

General Methods.-Marine sponge taxonomic identification was performed in the Smithsonian Institution where voucher specimens are deposited in the collections of the Department of Invertebrate Zoology, National Museum of Natural History. All solvents employed were redistilled. Size exclusion chromatography was accomplished with Sephadex LH-20 (Particle size: 25-100 um) supplied by Pharmacia Fine Chemicals, Uppsala Sweden. Thin-layer chromatography was carried out with silica gel CHLF Uniplates (Analtech Inc.) and with RP-8 precoated plates (layer thickness: 0.25 mm) from E. Merck, Darmstadt, Germany. High-speed countercurrent chromatography was accomplished with an Ito Multilayer Coil Extractor-Separator (P.C. Inc., Potomac, MD) using 2.6 mm I.D. tubing, and a FMI Lab Pump.

Melting points are uncorrected and were determined on a Kofler-type hot-stage apparatus. Ultraviolet spectra were recorded employing a Hewlett-Packard Model 8450A uv/vis spectrophotometer and ir spectra with a Nicolet ft-ir Model MX-1 instrument. Nmr spectra were measured in DMSO-d_6 using a Bruker AM-400 instrument and are recorded in ppm downfield to TMS (assignments bearing the same superscript may be reversed). The ^{13}C nmr multiplicities were determined with APT experiments based on an average coupling constant of 135 Hz. Eims spectra were recorded with a Kratos AEI 5076 spectrometer at the NSF Regional Facility, University of Nebraska, Lincoln, Nebraska.

Palau Porifera (Axinella sp. and Hymeniacidon sp.) and Extraction. The initial collection of Axinella sp. (Demospongiae class, Axinellida order, Axinellidae family) in Palau, Western Caroline Islands was conducted in May, 1979. The sponge displayed a brownish-yellow exterior of irregular mass. A 2-propanol-$CHCl_3$ extract gave confirmatory in vivo activity with PS T/C 201 at 100 mg/kg, PS ED_{50} = 2.5 µg/ml. A scale-up recollection (220 kg wet wt) of

this sponge was completed in March, 1985 and preserved in 2-propanol. The preserving solution was separated from the sponge, concentrated to an aqueous slurry and extracted with CH_2Cl_2 (13). The remaining sponge material was re-extracted with 2-propanol-CH_2Cl_2 (1:1); the extract was separated, solvent removed and the residue partitioned between CH_2Cl_2-H_2O. At this early stage a solid precipitate appeared at the CH_2Cl_2-H_2O interface. The precipitate was separated and amounted to 1.8 kg (PS T/C 227 at 294 mg/kg, ED_{50} 2.8 μg/ml). Analogous solid fractions were also obtained at this initial CH_2Cl_2 step during separations of the sponge extracts summarized below.

The orange sponge Hymeniacidon sp. (Demospongiae class, Halichondrida order, Hymeniacidonidae family) was collected (1979), recollected (1985) (218 kg wet wt.) and extracted (2-propanol extract showed PS T/C 130 at 5.5 mg/kg and ED_{50} 27 μg/ml) as summarized above. Removal of solvent from the initial 2-propanol extract led to an aqueous concentrate that contained 1.2 kg of a solid fraction with PS ED_{50} 3.7 μg/ml.

Isolation of Hymenialdisine (1b) and Axinohydantoin (2). A 10 g aliquot from the 1.8 kg of solid precipitate noted above was dissolved in CH_3OH (400 ml) and separated by size exclusion chromatgraphy on a column of Sephadex LH-20 (100 x 10 cm) to yield two major alkaloid fractions. When fraction 1 (elution volume: 12.0 - 12.6 liters) was allowed to stand for 24 h at room temperature, axinohydantoin (2) slowly crystallized as yellow needles (30 mg): mp > 350°; tlc on silica gel R_f = 0.83, 1-BuOH-AcOH 50% (95:5), tlc on RP-8 R_f = 0.51, CH_3OH-AcOH 5% (1:1); uv (CH_3OH) λ_{max} 264sh (log ε = 3.88), 345 (log ε = 4.16) nm; ir (KBr) ν_{max} 1740, 1702, 1638, 1480, 1425, 1407 cm^{-1}, ^1H nmr (DMSO-d_6) δ 2.67 (2H, m, H-6), 3.22 (2H, m, H-5), 6.66 (1H, s, H-14), 7.89 (1H, t, HN-4), 9.83, 10.91 (2x 1H, s, HN-9, HN-11), 12.35 (1H, s, HN-1); ^{13}C nmr (DMSO-d_6) δ 36.2 (t, C-6), 38.5 (t, C-5), 101.6 (s, C-15), 113.9 (d, C-14),

120.0 (s, C-13), 121.2 (s, C-7), 125.5 (s, C-2), 126.5 (s, C-8), 153.8 (s, C-10), 162.7 (s, C-3), 163.3 (s, C-12); hreims m/z 325.9842 and 323.9834 ($C_{11}H_9N_4O_3Br$ requires 325.9836).

Fraction 2 (elution volume: 12.9 - 13.5 liters) yielded a crystalline precipitate (100 mg) which was identified as hymenialdisine (1b) by comparison (uv, ir, ^1H nmr, eims) with an authentic sample (4).

Truk Porifera (Axinella carteri) Collection and Extraction.-In May 1985, approximately 1 kg of an orange-yellow sponge subsequently identified as Axinella carteri (Dendy), was collected in the Truk Lagoon, Federated States of Micronesia, at -13 to -24 m. The preserving solution (2-propanol) was removed and this extract proved toxic down to 50 mg/kg against the PS leukemia. The 2-propanol extract was partitioned between CH_2Cl_2 and H_2O and the resulting CH_2Cl_2 extract was successively partitioned (13) between 9:1 - 4:1 - 1:1 MeOH:H_2O with hexane - CCl_4 - CH_2Cl_2. The final CH_2Cl_2 extract showed PS T/C 135 at 100 mg/kg and PS cell line ED_{50} 1.2 µg/ml.

In October 1985, approximately 148 kg (wet wt) of the sponge was recollected and preserved in MeOH. The MeOH solution was decanted, and the sponge was ground and extracted with MeOH:CH_2Cl_2 (1:1). The original MeOH solution was concentrated to an aqueous phase and extracted with CH_2Cl_2 (3x) followed by 1-BuOH. Study of this 1-BuOH fraction was discontinued when PS results showed minimal activity.

When the ambient temperature extraction of the sponge with MeOH:CH_2Cl_2 was completed, the aq. MeOH phase was separated and concentrated to an aqueous phase which was extracted with 1-BuOH (15 liters). The 1-BuOH phase was concentrated, redissolved in MeOH (1.5 liters) and dried to give a 232 g fraction (PS ED_{50} = 1.4 µg/ml). A 97 g aliquot of the MeOH soluble fraction was treated with 1-BuOH (800 ml, 50°C, 12 hr) and the relatively insoluble part (50 g, PS ED_{50} = 1.5 µg/ml) was collected. The MeOH (600 ml) sparingly

soluble portion weighed 4.26 g (PS ED_{50} = 0.11 µg/ml).

Papua New Guinea Porifera (Hymeniacidon sp.) Collection and Extraction.- The collection (May 1981, near Motapure Island, Papua New Guinea) and recollection (October 1983, 44 kg wet wt) of an orange Hymeniacidon sp. as well as the large scale extraction (crude extract PS T/C 136 at 100 mg/kg and ED_{50} 24 µg/ml) and solvent partitioning was performed as just described above for A. carteri. In this case when the 934 g initial CH_2Cl_2 fraction was subjected to further separation by the MeOH-H_2O with hexane → CCl_4 → CH_2Cl_2 sequence a total of 135 g (PS ED_{50} 14 µg/ml) of a solid interfacial fraction was collected and used to isolate hymenialdisines 1a and 1b.

Isolation of Hymenialdisines 1a and 1b.- Procedure A. An aliquot (250 ml) of the preceding Axinella carteri MeOH (600 ml) solution was applied to a column of Sephadex LH-20 (1.9 kg in MeOH). A total of 460 fractions of 20 ml each were collected and a fraction weighing 0.73 g (PS ED_{50} 2.2 µg/ml) was further separated using high speed countercurrent distribution with an Ito coil. A 50 mg aliquot was applied (6 ml) in 1-BuOH:HOAc:H_2O (4:1:5) to the coil with the 1-BuOH phase as stationary (upper) and the aqueous part as mobile (lower) phase. Fractions (120) of 6.5 ml each were collected; fractionation was monitored with UV detection (254 nm). The fractions were neutralized (pH 7) with aq NaOH and refrigerated. Debromohymenialdisine 1a, 9 mg, PS ED_{50} = 3.0 µg/ml, crystallized from fractions 28-33 and was identical (TLC, MS, NMR) with an authentic sample (6).

The MeOH less soluble fraction (4.26 g) described above was extracted with MeOH (5 x 25 ml) at 40°C and the solution filtered to give 3.73 g of residue. A 0.90 g portion was triturated with DMSO (10 ml). The soluble portion (0.25 g) was chromatographed on a column of Sephadex LH-20 in MeOH to provide 0.13 g of hymenialdisine (1b) as yellow crystals (PS ED_{50} = 0.62

µg/ml) identical (tlc and ms comparisons) with an authentic sample (6).

Procedure B.-The 1.2 kg fraction (see above) from the Palau Hymeniacidon sp. was further separated by successive soxhlet extraction (20 g aliquot) with CH_2Cl_2 (6 x 5 liters), EtOH (6 x 5 liters) and 1-BuOH (6 x 5 liters) to give respectively 35 g (PS ED_{50} = 26 µg/ml), 500 g (PS ED_{50} 8.6 µg/ml) and 106 g (PS ED_{50} = 2.6 µg/ml) fractions. A 10 g sample of the 1-BuOH fraction in MeOH was subjected to chromatography on a column of Sephadex LH-20 (500 g) to give 26 individual (by tlc comparisons) fractions using 4:1 CH_2Cl_2-MeOH. Of these, 56 mg proved to be largely debromohymenialdesine 1a (PS ED_{50} 1.4 µg/ml) and hymenialdesine (5.5 mg, 1b, PS ED_{50} 7.5 µg/ml) by comparison nmr and tlc.

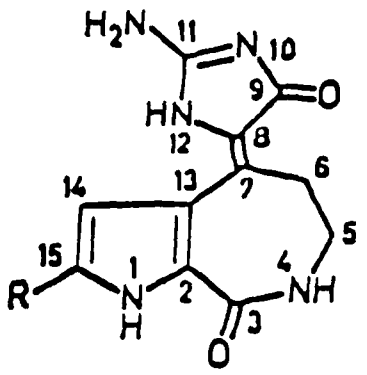
Procedure C.-The 135 g fraction from the Papua New Guinea Hymeniacidon sp. was extracted (Soxhlet procedure with two stainless steel 1-gallon extractors) with EtOH to yield a 22 g alcohol soluble fraction. Treatment of this fraction with CH_2Cl_2-CH_3OH (1:1) yielded a precipitate (4.3 g, PS ED_{50} 8.5 µg/ml) which was extracted with hot 1-BuOH. The 1.5 g 1-BuOH soluble fraction was preabsorbed onto silica gel and separated by chromatography on a column (3 x 62 cm) of silica gel (180 g). Gradient elution with 95:5 CH_2Cl_2-CH_3OH with increments of MeOH provided fractions that yielded (0.17 g and 0.06 g respectively) debromohymenialdisine (1a, PS T/C 143 at 3.6 mg/kg and ED_{50} 2.5 µg/ml) and hymenialdisine (1b, PS T/C 138 at 3.6 mg/kg and ED_{50} 2.7 µg/ml). Both 1a and 1b were identified by direct comparison with authentic samples (6) employing tlc, ^{13}C and ^{1}H-nmr, ms, uv and ir spectral data.

ACKNOWLEDGMENTS

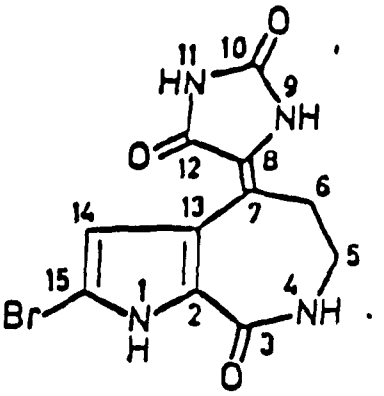
We are pleased to acknowledge the very necessary financial assistance provided by the Fannie E. Rippel Foundation, the Arizona Disease Control Research Commission, the Robert B. Dalton Endowment Fund, Virginia Piper, Polly Trautman, Eleanor W. Libby, the Donald Ware Waddell Foundation, Herbert and Dianne Cummings, (The Nathan Cummings Foundation, Inc.), Mary Dell Pritzlaff, the Olin Foundation (Spencer T. and Ann W.), PHS Grants CA-16049-07-12 awarded by the National Cancer Institute, DHHS, and by the U.S. Army Medical Research and Development Command under Grant No. DAMD17-89-Z-9021. Other helpful assistance was provided by the Governments of Papua New Guinea (N. Kwapena, A. Richards, and Drs. J. M. Lock and N. Polunin), the Federated States of Micronesia (Truk, D. E. Aten, R. Killion and A. Amaraich) and Palau, (Dr. T. Paulis and K. B. Batcheller), Singapore Airlines Ltd. (M. Theam Kong), Blue Lagoon Dive Shop (MOEN, Truk) Drs C. G. Bass, C. Dufresne, G. R. Pettit III, D. K. Tackett, L. P. Tackett, S. Taylor, T. N. Trautman and the Smithsonian Institution Oceanographic Sorting Center, The U.S. National Science Foundation (Grant CHE-8409644), The Swiss National Science Foundation (to D.E.S.) and the NSF Regional Instrumentation Facility in Nebraska (Grant CHE-8620177).

LITERATURE CITED

1. J. A. McBain, G. R. Pettit, and C. C. Mueller, Carcinogenesis, submitted for publication.
2. G. R. Pettit, J. F. Day, J. L. Hartwell, and H. B. Wood, Nature, 227, 962 (1970),
3. Y. Kato, N. Fusetani, S. Matsunaga, and K. Hashimoto, J. Org. Chem., 53, 3930 (1988).
4. Y. Hirata and D. Uemura, Pure & Appl. Chem.., 58, 701 (1986).
5. G. Cimino, S. DeRosa, S. DeStefano, L. Mazzarella, R. Puliti, and G. Sodano, Tetrahedron Letters, 23, 767 (1982).
6. I. Kitagawa, M. Kobayashi, K. Kitanaka, M. Kido, and Y. Kyogoku Chem. Pharm. Bull., 31, 2321 (1983).
7. F. J. Schmitz, S. P. Gunasekera, V. Lakshmi, and L. M. V. Tillekeratne, J. Nat. Prod., 48, 47 (1985).
8. P. Main, S. J. Fiske, S. E. Hull, L. Lessinger, G. Germain, J. P. Declerq and M. M. Woolfson, MULTAN 80. A System of Computer Programs for the Automatic Solution of Crystal Structures from X-Ray Diffraction Data. University of York, England, and Louvain, Belgium.
9. L. Chevolot, S. Padua, B. N. Ravi, P. C. Blyth, and P. J. Scheuer, Heterocycles, 7, 891 (1977).
10. R. Kazlauskas, P. T. Murphy, R. J. Quinn, and R. J. Wells, Tetrahedron Letters, 1, 61 (1977).
11. P. Djura, D. B. Stierle, B. Sullivan, D. J. Faulkner, E. Arnold, and J. Clardy, J. Org. Chem., 45, 1435 (1980).
12. D. J. Faulkner, Nat. Prod. Reports, 3, 1 (1986).
13. G. R. Pettit, Y. Kamano, R. Aoyagi, C. L. Herald, D. L. Doubek, J. M. Schmidt and J. J. Rudloe, Tetrahedron, 41, 985 (1985).



1a, R = H debromohymenialdisine
1b, R = Br hymenialdisine



2
axinohydantoin

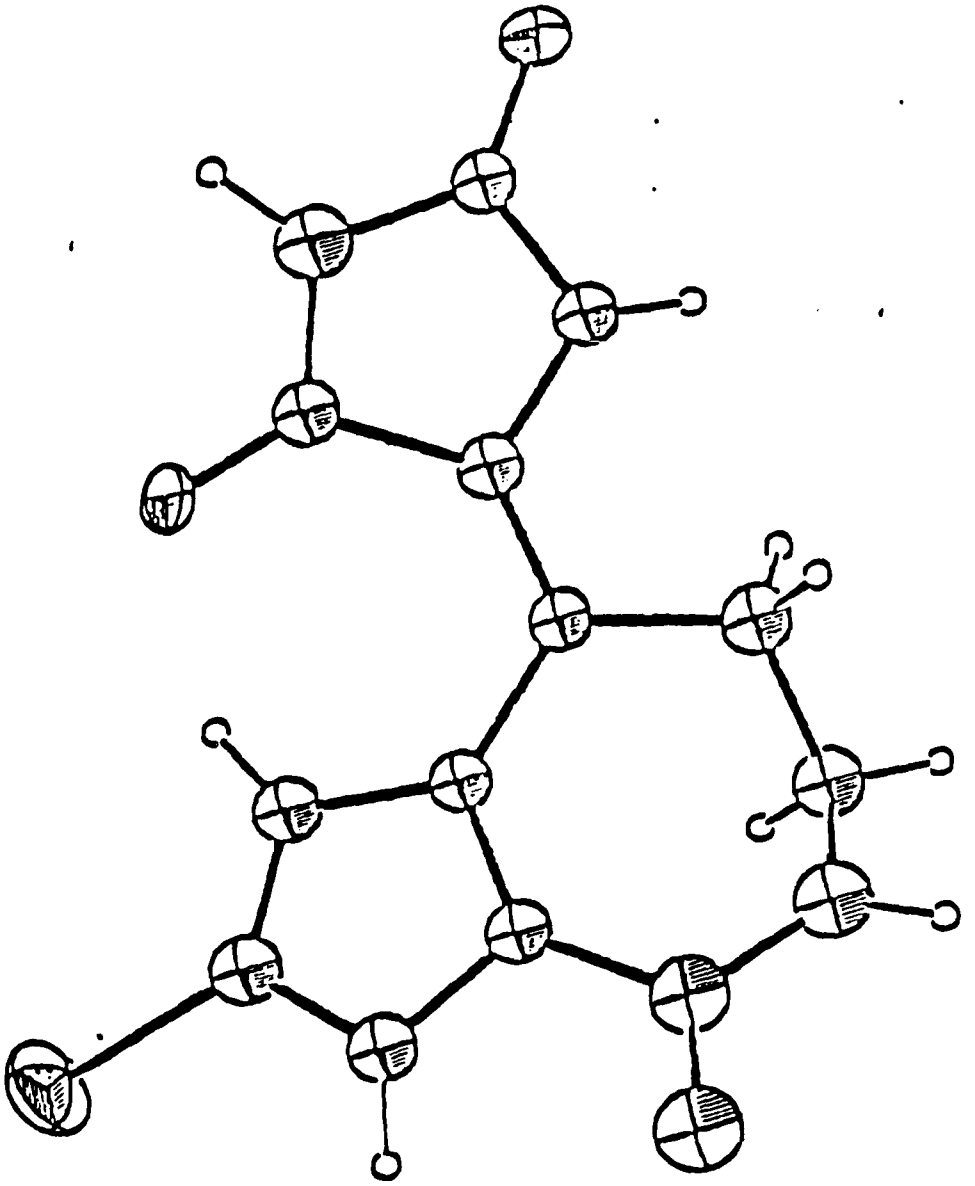


Figure 1. ORTEP view of a single molecule of 2, with 50% thermal ellipsoids.

TABLE 1

X-ray Crystal Structure Determination of Axinohydantoin (2)

Crystal Data

$C_{11}H_8BrN_4O_3 \cdot CH_3OH$
F.W. 357.17
F(000) = 1440
crystal dimensions: 0.17 x 0.13 x 0.08 mm
peak width at half-height = 0.21°
Nb filtered Mo Kα radiation (λ = 0.71073 Å)
temperature = 23°±1
monoclinic space group C2/c
a = 19.558 (2) Å b = 7.505 (1) Å c = 19.092 (3) Å
β = 103.78 (1)°
V = 2754.3 Å^3
Z = 8
ρ(calc) = 1.72 g/cm^3
μ = 29.7 cm^{-1}

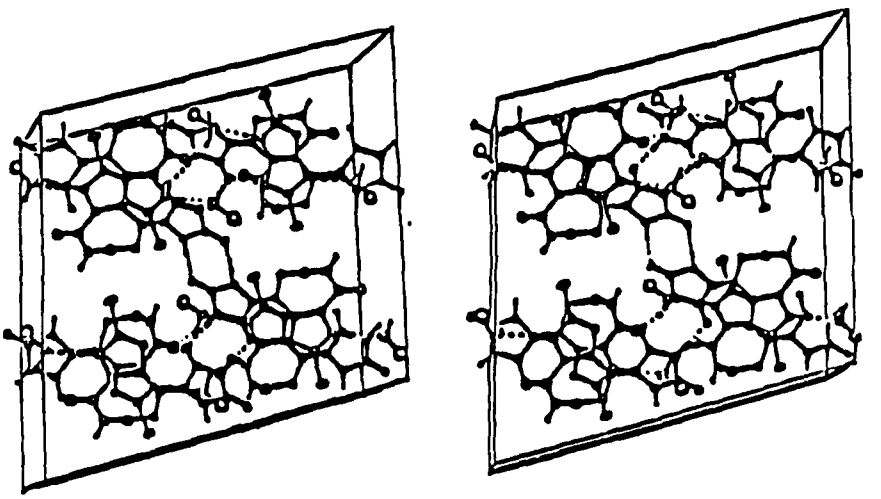
Intensity Measurements

Instrument: Nicolet P2$_1$
Scan type: 2θ/θ
Scan rate: 1.5-6.0°/min
Scan range, deg: $2.0 + (2\theta_{K_{\alpha_2}} - 2\theta_{K_{\alpha_1}})$
Maximum 2θ: 50.0°
No. of refl. measured: 2914 total, 2446 unique
Corrections: Lorentz-polarization
Reflection averaging (agreement on I = 2.6%)
Numerical absorption (from 07.41 to 75.48 on I)

Structure Solution and Refinement

Solution: Direct methods
Minimization function: $\Sigma w(|Fo| - |Fc|)^2$
Least-squares weights: $4\,Fo^2/\sigma^2(Fo^2)$
Anomalous dispersion: All non-hydrogen atoms
Reflections included: 836 with $Fo^2 > 3.0\sigma(Fo^2)$
Parameters refined: 105
Unweighted agreement factor: 0.053
Weighted agreement factor: 0.055
Esd of obs. of unit weight: 1.29
Convergence, largest shift: 0.02
High peak in final diff. map: 0.42 (10) e/Å^3
Computer hardware: PDP-11
Computer software: SDP-PLUS (Enraf-Nonius)

SUPPLEMENTARY MATERIAL



ORTEP stereoview of a unit cell of 2 with a vertical. Hydrogen bonds are shown as dashed lines.